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FRONTIERS IN CYTOLOGY

Frontiers in Cytology

EDITED BY SANFORD L. PALAY

New Haven: Yale University Press, 1958

Frontispiece: Hepatic parenchymal cell.
Electron micrograph by Dr. Keith R. Porter

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The following studies are based on a series of lectures presented at Yale University and dedicated to the memory of Henry Bunting

cedures for fixing, embedding, and sectioning tissues, it has become possible to visualize directly structures having diameters of 20 to 30 A. Although the current procedures have been in use only about three years, much new information has been collected. In our laboratory we have been particularly interested in several membranous structures within cells, especially plasma membranes, mitochondria, and ergastoplasm. The structure of these elements varies from one location to another, and in different physiological states. Indeed, such profound modifications can be induced by experimental procedures designed to increase or decrease cellular activities that it would seem we are observing the actual mechanisms involved in absorption, synthesis, and secretion.

PLASMA MEMBRANES. With the light microscope, the limiting plasma membranes of cells appear sometimes as a dense pellicle and sometimes as a wavy indistinct line which frequently fades into invisibility. In tissue cultures of fibroblasts, for example, a stable and quiescent region of the cell membrane may be highly refractile, whereas the margin of an actively moving pseudopodium, particularly at its tip, may be so indistinct as to verge on invisibility. Similar variability is readily observed in electron micrographs. Here, however, the thick, dense regions have an appearance suggesting an adsorbed layer of dense, homogeneous material upon a delicate membrane, which in other locations apparently lacks such apposed material and so appears much more tenuous.

The plasma membranes separating one cell from another vary greatly in their complexity. In some locations, such as the sides of many prismatic epithelia, the membranes exhibit plane surfaces. In others, for example in the yolk-sac epithelium of the guinea pig, occasional projections from one cell fit accurately into corresponding recesses in its neighbor (Fig. 8). Still other regions may be characterized by a complex dovetailing, as occurs between the chorionic and uterine epithelia of the horse's placenta.

The apical borders of cells lining cavities exhibit elaborate modifications of their limiting membranes. Only rarely do they present a smooth and regular surface. The usual situation involves finger-like projections or microvilli which may be infrequent, as in endothelial cells; numerous and irregular, as in the human placental syncytium (Fig. 4); numerous and regular, as in the absorptive cells of the small intestine; short, as in the thyroid follicle (Fig. 12); and elongated and occasionally bulbous, as in the yolk-sac epithelium (Fig. 1). Because such microvilli occur regularly in cells exhibiting an absorptive function, they were at first

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Contents

Contributors vii
Foreword xi
1. *Henry Bunting,* BY WILHELM S. ALBRINK 1
BIBLIOGRAPHY 6
2. *Current Concepts of Cellular Structures,* BY E. W. DEMPSEY 9
3. *Structural Specializations of the Cell Surface,* BY DON W. FAWCETT 19
4. *Chromosomes: Their Constitution and Function,* BY A. R. T. DENUES 42
5. *Studies on Mitosis in Purine-Treated Tissue Cultures,* BY JOHN J. BIESELE 84
6. *Changes in the Desoxyribonucleoprotein Complex during the Cell Cycle,* BY DAVID P. BLOCH 113
7. *Pentosenucleic Acids in Relation to Nuclear and Cytoplasmic Functions,* BY JOHN I. NURNBERGER AND MALCOLM W. GORDON 167
8. *Intracellular Lipides: Their Detection and Significance,* BY HELEN WENDLER DEANE 227
9. *Some Aspects of Protein Histochemistry, with Special Reference to Protein Hormones,* BY RUSSELL J. BARRNETT 264
10. *A Small Particulate Component of the Cytoplasm,* BY GEORGE E. PALADE 283
11. *The Morphology of Secretion,* BY SANFORD L. PALAY 305
12. *The Cytology of Striped Muscle,* BY H. STANLEY BENNETT 343
13. *Cell Transformation and Differentiation in Regenerating Striated Muscle,* BY GABRIEL C. GODMAN 381
14. *Pathological Swelling and Vacuolization of Cells,* BY ELIAS E. MANUELIDIS 417
15. *Cellular Reaction during Virus Infections,* BY WILLIAM H. GAYLORD, JR. 447
16. *The Dermal Ground Substance of the Mesenchyme as an Element of Natural Resistance against Infection and Cancer,* BY F. DURAN-REYNALS 472
17. *Collagen and Reticulin,* BY WILLIAM G. BANFIELD 504
Index 521
Plates follow Index

Foreword

THIS VOLUME arose out of a group of lectures given at Yale University in memory of the late Dr. Henry Bunting by his friends during the winter of 1955. In that year these lectures substituted for the elective course in analytical cytology that Dr. Bunting and the editor had given annually during the previous six years for medical students of the preclinical years. The manuscripts of the chapters were collected during 1956, and the authors had an opportunity to bring them up to date in the spring of 1957.

The past two decades have seen a remarkable resurgence of cytology, which has gained momentum in the present decade through the application of the new techniques of cytochemistry, histochemistry, and microscopy. Out of this recrudescence new concepts are developing which will certainly affect the fundamental principles of all other biological disciplines just as radically as the cell theory revolutionized biology more than one hundred years ago. Since the properties of life are inherent in the *organization* of living matter, cytology serves as a unifying science, bringing the disciplines of physiology and biochemistry together within the framework of morphology. In recent years numerous symposia have been held and many books have been published dealing with technical aspects of the new cytology, but no presentation has yet appeared of the new concepts and hypotheses growing out of the knowledge obtained with these techniques. The contributors to this volume hope that it will at least partially fulfill this need.

The scope of cytology is now too large to be presented in a single volume. The selection of topics included here does not necessarily reflect their current or ultimate importance, but rather the interest of the contributors and the state of the subject. Therefore, this book does not pretend to embrace the entire field of modern cytology. Furthermore, the thoughtful reader will find many contrary opinions expressed by the authors of the several chapters. These contradictions were not eliminated because lack of unanimity is characteristic of a developing subject, and in many instances more systematic study is necessary before firm decisions can be reached. Consequently, the reader will find in these chapters traces of the lively controversies which have always distinguished cytology in the past.

The publication of this volume was made possible by a generous grant (No. C-2618) from the National Cancer Institute, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, to which the editor and contributors are grateful. The editor wishes to express his sincere thanks to the staff of the Yale University Press for their continued interest in this book and for their assistance in its preparation.

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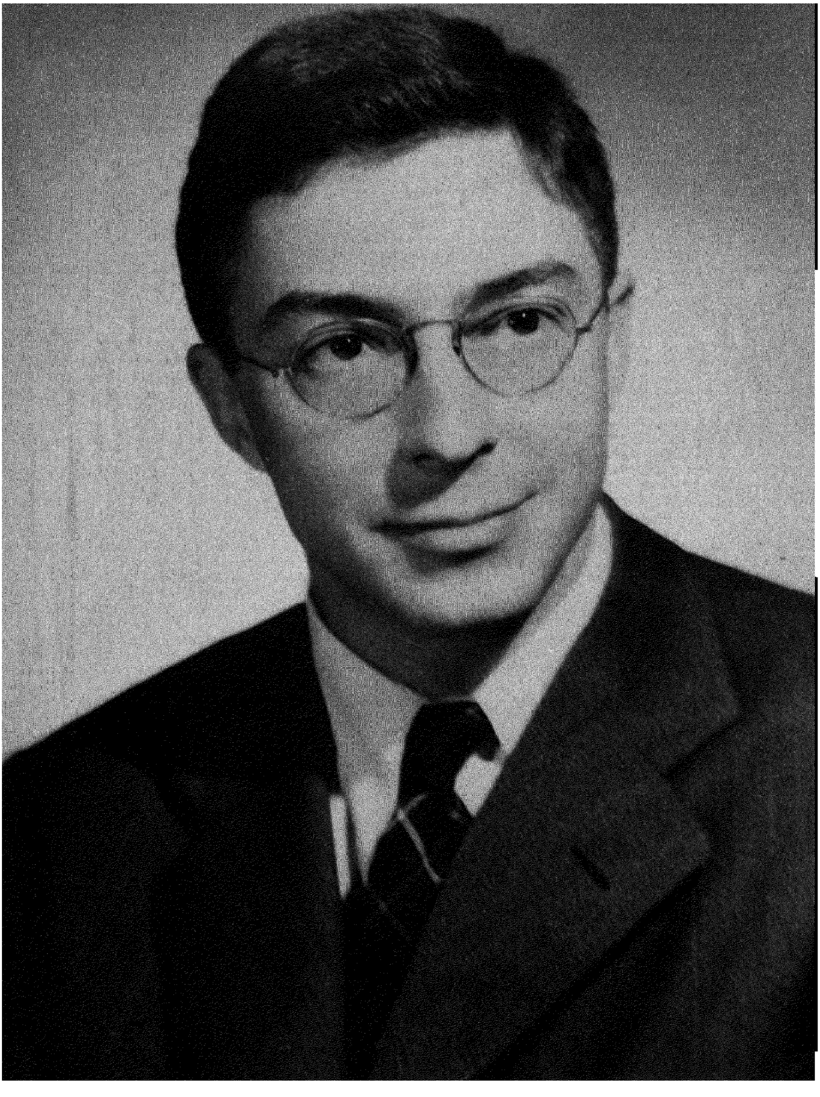

1. *Henry Bunting*

BY WILHELM S. ALBRINK

HENRY BUNTING was born March 10, 1911, in Chicago, Illinois, of New England heritage and into a home imbued with that tradition. His father, Charles Henry Bunting, Professor in Pathology at the University of Wisconsin, and Chairman of that Department, was of New England forbears, although he himself had been born in La Crosse, Wisconsin. His mother, Carlotta Sweat Bunting, was a native of Bangor, Maine, where she had practiced medicine with her father for ten years before her marriage. An older daughter, Elizabeth, completed the family.

As a member of this close-knit and self-sufficient family, Henry was satisfied with a few intimate childhood friends. In his father he found a kind and tolerant man with whom he formed a lasting friendship transcending familial ties and treasured by both father and son. Dr. Bunting imparted much of his own wit and wisdom to Henry during his developing years and, by example, helped formulate the traits of justice and consideration for others so characteristic of the adult Henry. He later impressed his son, the medical student, with the necessity for thoroughness of observation, clarity of thought, and honesty of conclusion. In return he received from Henry the affection of a devoted son, the admiration of a student for a revered teacher, and the appreciation of a man for a friend. A long-standing ambition was realized for each when they published a scientific paper together [45].

Although Henry was always an excellent student, he used books not as a means of escape but rather as instruments for reference or aesthetic enjoyment. He preferred active pursuits to passive ones, a characteristic which persisted throughout his life. Thus he learned silvercraft and flute playing in preference to reading the classics, and he delighted in observing birds and animals in their natural environment. His interest and proficiency in bird watching enabled him to gain a bird banding license at sixteen and provided him with one of his favorite occupations for the rest of his life. He had also a keen interest in beetles and at an early age began his extensive collection, which is now in the Peabody Museum of Natural History in New Haven.

After graduating from high school in Madison, Henry, deemed by his parents too young to go to college, spent a year at Andover. He then entered the University of Wisconsin for two years and transferred to Yale for his final two years of college work. While at Wisconsin he had been interested in astronomy and had become a variable-star observer. He pursued his study of the stars at Yale and secured a job showing people through the observatory. The money which he earned at this pleasant task was used to purchase his microscope for medical school. He continued his interest in animals by taking courses and special work in the Zoology Department.

Following the completion of his undergraduate degree at Yale, Henry returned to the University of Wisconsin, where he spent the next two years taking his preclinical work in Medical School and earning his Master's degree in Physiology. The investigations comprising his Master's thesis formed the basis for his first published paper [1].

Of even greater significance than the beginning of his laboratory investigative career was the fact that during this period, Henry met his future wife, Mary Ingraham, better known as Polly, a Brooklyn girl who was doing postgraduate work in bacteriology at the University. Polly Ingraham possessed many qualities in common with Henry: a love of nature, a superior intellect, sound common sense, and a driving energy even greater than his own. They were not married until after Henry had served a year's internship in medicine at Johns Hopkins Hospital following his graduation from Harvard Medical School.

In 1938, after completing an assistant residency at Baltimore City Hospital, Dr. and Mrs. Bunting went to Yale, he as a Childs Fellow in Pathology and she as a research assistant in bacteriology. Henry performed autopsies and studied slides along with the house staff as well as conducting research on cancer in mice [3–6]. The Buntings had found a small house in the rural community of Bethany. Here they were able to keep bees, raise chickens and goats, and enjoy an outdoor life so important to both. They continued living in Bethany and playing an integral part in the community's affairs for the remaining sixteen years of Henry's life. In this environment Henry unveiled and developed an outgoing friendliness and love of people which became one of his most outstanding virtues.

In 1941 the Committee on Medical Research, Office of Emergency Management, negotiated a contract with Yale University to study means of treatment of war-gas casualties. Dr. M. C. Winternitz, then Chairman of the Pathology Department, was the responsible investigator, and he delegated the primary responsibility to Henry, then an instructor in the department. Later Henry was promoted to assistant professor and became the responsible investigator. The involvement of this country in actual war and its inroads into the teaching staff of the Pathology Department placed a heavy load on his shoulders. It was at this time that he utilized his almost fantastic ability to do several things at one time and to do them well. As Dr. Nelson K. Ordway, one of his co-workers at that time has expressed it, "he would simultaneously look through a microscope at pathologic dog lung sections, eat with his left hand, write with his right hand, read a copy of *Science* open in front of him with the eye not occupied through the microscope, and go through in his mind the organization of his lecture for the following day for second-year medical students—all this while listening to us relate to him the morning's activities with our gassed dogs." This hard-driving pace took its toll, however, and Henry was forced to retire to a tuberculosis sanatarium for a few months.

During the course of this contract the possibilities of using histochemical methods to extend morphological observations were made manifest [10, 13], and after the war he spent a year as a Commonwealth Fellow in study at the Massachusetts Institute of Technology and in the Department of Anatomy at the Harvard Medical School. It was during this year that his diversified pursuits began to converge on a discipline that would yield both scientific and personal satisfaction. In analytical cytology could be merged the patience of the bird watcher, the meticulous attention to detail of the silver craftsman, the observational powers of the naturalist and astronomer. And this discipline could be applied to the broad biological problems presented by pathology.

With Drs. G. B. Wislocki and E. W. Dempsey of the Anatomy Department of Harvard Medical School, Henry began his study of the noncellular constituents of connective tissues, which resulted in the publication of two papers concerning the histochemical properties of mammalian mucopolysaccharides [16, 18]. Although he investigated other structures by histochemical means during this period, the problem of the mucopolysaccharides was the most intriguing one to him and represented one of the two major lines of research which he followed on his return to Yale. The basis for the other major project was established during the period of study at MIT. Here Henry became familiar with the newer tools of microscopy, including the electron microscope. This knowledge permitted his subsequent investigations on the role of viruses associated with certain benign cutaneous neoplasms.

In 1947 Henry returned to Yale and began the most active period of his life. Now that he had determined his field of interest in pathology and had time to pursue it, he began at once to apply his newly developed skills. Thus he was able to solve many small vexing problems about the character of minute alterations in structure which he had observed in routine pathological examinations. Although many of these were not of sufficient importance of themselves to warrant publication, they added to his already considerable store of knowledge and increased his efficacy as a teacher. And these problems could be pursued with relatively simple equipment while he began the fascinating but laborious process of assembling the tools of a modern histochemical laboratory.

Before his own electron microscope could be ordered and installed, Henry used one in the section of Preventive Medicine. He and his good friend, Dr. Maurice Strauss, the dermatologist of the Yale Medical School, began a study of skin papillomas. They and their collaborators were able to compare and contrast skin papillomas of various types by means of histochemical techniques and to demonstrate the formation of viruses within the cell by the use of the electron microscope on thin sections [29, 33–6, 42–4].

Henry was also busy with the teaching program. It was his responsibility to arrange schedules and to provide substitutes when needed, a difficult job because much of the instruction was carried out in groups of six or eight students. Henry himself took over many of the lecture subjects, including some which had no intrinsic interest for him. But his sincere interest in the student drove him to do a more creditable job than would others. On the lecture platform he was informal and shunned the didactic approach often exhibited by teachers, particularly of pathology. In small sessions with students, house officers, or colleagues he came into his own as a teacher, for here he would listen carefully and respond with kindness and wisdom, never with impatience or ridicule. He seemed never too busy to listen to problems, both personal and professional, presented to him by students, colleagues, or others who knew him, and to help in any way that he could, regardless of personal sacrifice.

This spirit of helpfulness was not confined to his place of work or to his professional associates but was very apparent in the community where he lived, for he responded to the calls of those who needed medical help no matter when the call came. He seemed equally available to help in moving furniture, building a fence, or cleaning paint from the windows of a new house.

From an early age the Bunting children, Mary, Charles, William, and John, were given partnership rights in the family affairs and responsibilities commensurate with their abilities. They could always rely on parental encouragement for their individual projects and hobbies, and on actual assistance when it was indicated. They constantly had before them two examples of good humor and could not help following these examples. From their parents they learned to expect kindness and sympathy but not sentimentality, to receive considered appraisals rather than dramatizations of seeming misfortunes.

Henry still pursued many of his earlier hobbies whenever his time allowed. He loved to watch the migration of birds and would drive miles to spend two or three days participating in a bird census. He played flute in the Business and Professional Men's Symphony Orchestra and in occasional informal ensembles gathered in someone's home for this purpose. He taught himself to play the guitar and would on request bring it to parties to accompany the singing of folk songs. He was a favorite guest because of his quick wit and contagious good humor.

At the time of his unexpected death on April 15, 1954, after a short illness, Henry Bunting had achieved unusual stature among his fellowmen. He was an associate professor of pathology at Yale, with the major responsibility of teaching the medical students. Many scientific publications not bearing his name had benefited by his careful scrutiny of them and his considered suggestions about them. His qualities of intellectual honesty, meticulous attention to detail, and freedom from cant had influenced students and colleagues alike. His numerous scientific papers had been accepted as sound contributions and indicated that others of even more significance would be forthcoming. The community in which he lived respected him as a leading citizen and a good neighbor. The universal affection in which he was held was emphasized by the attendance at a memorial service for him held in Dwight Chapel on April 22, 1954. The crowded chapel heard an eloquent tribute by his lifelong friend, Thomas C. Mendenhall, Master of Berkeley College, of which Henry was a fellow.[1] Those present included undergraduates, medical students, former patients, neighbors, workmen who had helped build his house, fellow musicians and bird watchers, and professors, all having but one thing in common—a shocked sense of loss because a valued friend had died.

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[1] Reprinted in Yale J. Biol. Med., *26,* 422, 1953–54.

These friends have established memorials to Henry Bunting, and it is proper that they did so. But during his lifetime he had established by his own wisdom, love of people, and generosity of time the most fitting memorial of all, a heterogeneous group of people who are better for having known him.

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2. *Current Concepts of Cellular Structures*

BY E. W. DEMPSEY

1. Histochemistry—Specificity vs. Localization

HISTOCHEMICAL OPERATIONS are in no sense new inventions. Ehrlich [10], Mann [17], and others were much interested in the chemical composition of the structures visible with the aid of the microscope. However, neither analytical chemistry nor biochemistry had progressed far enough in their days to afford more than a spotty beginning. In more recent years, dating most readily from the classical work of Lison [16], a more systematic and sustained attack has been possible. Procedures are now available for defining and differentiating several varieties of lipides, many kinds of carbohydrates, and a rather large number of proteins, including the nucleoproteins, the sulfur-rich proteins, proteins with specific immunochemical properties, and a host of enzymes. These procedures have varying degrees of utility; some are highly specific chemically and exhibit precise morphological localization, whereas others leave much to be desired. Nevertheless, with them it has been possible to further greatly our understanding of the processes whereby cells elaborate the highly individual substances utilized in secretion and differentiation. Countless examples could be cited; a few will suffice.

The lipides found in the adrenal cortex, ovary, testis, and placenta are characteristic in their reactions and fluctuate in amounts in ways which can be correlated with the physiological activities of the organs. Nucleoproteins, identifiable by their basophilia, their ultraviolet absorption spectrum, and their individual susceptibility to enzymatic hydrolysis, have been differentiated into two classes. One of these, the desoxypentose nucleoproteins, occurs almost exclusively in the nuclei of cells, and undergoes alterations which may be correlated with the mitotic rhythm and with genetic phenomena. The other class, the pentose nucleoproteins, occurs in both nucleus and cytoplasm and appears to be related to the cell's synthetic capacities. Proteins, the sulfur content of which is responsible for their physical or chemical identity, can be localized in structures such as hair and skin and in the secretory granules of the pancreas and hypophysis. Finally, a large number of enzymes have been identified in cells, one group of which seems to be largely concentrated in the mitochondria. It follows from all of this that the cell does not participate as an amorphous whole in its manifold activities, but that it contains a series of compartments, each of which exhibits a characteristically related structure, function, and composition. The segregation of the cell's activities among its organelles may be compared to the partition of function among the organs of the entire individual.

A preoccupation with the problem of intracellular localization of functions distinguishes the chemical histologists, or cytologists, from the microchemists. Basically, both morphologists and biochemists are concerned with the chemical composition of cellular elements. On the one hand, chemists have approached the problem by devising chemical methods which work on extremely small aliquots of tissue. An armamentarium of such methods is now available for the quantitative study of samples as small as a single cell, but these methods give little information concerning the intracellular, or cytological, localization of substances. On the other hand, microscopists have devised qualitative reactions which are moderately well localized within the cell, but these procedures frequently lack quantitative accuracy. Between these two methodologies lies an investigative field which, when fully explored, will form a continuum between chemistry and morphology. Lest anyone be discouraged from entering this field because he believes the gap will be quickly closed, however, a few calculations will indicate that a tremendous amount of work needs to be done. The most sensitive of the microchemical methods can deal with amounts of material equivalent to those contained in a cube the sides of which are about 10 μ. The light microscope can resolve structures the linear diameters of which are one-hundredth of this, or 0.1 μ. With the electron microscope, another hundredfold increase in resolving power is possible, so that structures 0.001 μ or 10 A in diameter may be visualized.

2. *Submicroscopic morphology*

For many years indirect methods, such as X-ray diffraction and polarized light, have been employed with the aim of deducing the presence of structures too small to be seen with the ordinary microscope. With the introduction of electron microscopy together with auxiliary procedures for fixing, embedding, and sectioning tissues, it has become possible to visualize directly structures having diameters of 20 to 30 A. Although the current procedures have been in use only about three years, much new information has been collected. In our laboratory we have been particularly interested in several membranous structures within cells, especially plasma membranes, mitochondria, and ergastoplasm. The structure of these elements varies from one location to another, and in different physiological states. Indeed, such profound modifications can be induced by experimental procedures designed to increase or decrease cellular activities that it would seem we are observing the actual mechanisms involved in absorption, synthesis, and secretion.

PLASMA MEMBRANES. With the light microscope, the limiting plasma membranes of cells appear sometimes as a dense pellicle and sometimes as a wavy indistinct line which frequently fades into invisibility. In tissue cultures of fibroblasts, for example, a stable and quiescent region of the cell membrane may be highly refractile, whereas the margin of an actively moving pseudopodium, particularly at its tip, may be so indistinct as to verge on invisibility. Similar variability is readily observed in electron micrographs. Here, however, the thick, dense regions have an appearance suggesting an adsorbed layer of dense, homogeneous material upon a delicate membrane, which in other locations apparently lacks such apposed material and so appears much more tenuous.

The plasma membranes separating one cell from another vary greatly in their complexity. In some locations, such as the sides of many prismatic epithelia, the membranes exhibit plane surfaces. In others, for example in the yolk-sac epithelium of the guinea pig, occasional projections from one cell fit accurately into corresponding recesses in its neighbor (Fig. 8). Still other regions may be characterized by a complex dovetailing, as occurs between the chorionic and uterine epithelia of the horse's placenta.

The apical borders of cells lining cavities exhibit elaborate modifications of their limiting membranes. Only rarely do they present a smooth and regular surface. The usual situation involves finger-like projections or microvilli which may be infrequent, as in endothelial cells; numerous and irregular, as in the human placental syncytium (Fig. 4); numerous and regular, as in the absorptive cells of the small intestine; short, as in the thyroid follicle (Fig. 12); and elongated and occasionally bulbous, as in the yolk-sac epithelium (Fig. 1). Because such microvilli occur regularly in cells exhibiting an absorptive function, they were at first thought to be a device for increasing the absorptive surface. However, they are also found on the surfaces of cells which are primarily secretory, such as the acinar cells of the pancreas and uterine glandular epithelium (Fig. 7). Such microvilli on apposed cells may interdigitate with one another, as for example in the horse's placenta, where the chorionic epithelium is fused with the uterine endometrium. In such attachments, microvilli from each cellular layer interdigitate with the other to form a union fancifully reminiscent of a zipper (Fig. 6).

The free surfaces of cells frequently exhibit another modification, in which the plasma membrane is invaginated inward rather than projected outward as is the case with microvilli (Fig. 2). In the yolk-sac epithelium of guinea pigs, occasional pores occur in the free surface, which communicate with a complex system of apical canaliculi. Some of the canaliculi, especially those extending farthest beneath the surface, are dilated. In these dilated canaliculi granular material occurs, the appearance of which is similar to that seen in the lumen. This system of pores, canals, and vacuoles has been likened to the gullet and food-vacuole of protozoa such as paramecia [5]. Presumably, therefore, the system is an absorptive one.

Similar canalicular systems, communicating with the cell's exterior through surface pores, have been encountered in diverse cells. In a specialized portion of the pig's chorion, the areola, extensive labyrinthine invaginations of the cell membrane occur [9]. In Schwann cells, neuronal processes are surrounded by sheathing cytoplasm, which maintains, nevertheless, a connection to the cell's exterior by means of a communicating pore and canal [12]. Glial cells in the central nervous system exhibit most complicated inward foldings of their surface membranes (Fig. 3). Neurons frequently possess canalicular channels which represent invaginations of the surface membrane. The hair cells of the internal ear display a complex outer layer, consisting of a relatively smooth plasma membrane underneath which are numerous minute vesicles. These vesicles sometimes become organized into bodies which extend deeply into the cytoplasm, where they form the bodies of Retzius and Hensen as described with the ordinary microscope. Studies in collaboration with Dr. Catherine Smith have indicated that these bodies and their component membranous vesicles are formed from inpouching extensions of the surface plasma membrane. In view of the variety of cells exhibiting complex, internal folds or invaginations of their plasma membranes, it would appear that we are dealing with a general potentiality of cell surfaces. For convenience, and by analogy with the term "microvillus," we have begun to describe these canalicular spaces as "microlabyrinths."

ERGASTOPLASM. With the introduction of electron-microscopic techniques which permitted observations of intracellular structures, attention was quickly drawn to a system of granular and membranous structures widely occurring in most types of cells. These intracellular membranes have been extensively studied by Palade [20], Porter [21], Bernhard, *et al.* [3], Dalton, *et al.* [4], and others. They have been called variously "lamellae," "endoplasmic reticulum," "intracellular membranes," and "ergastoplasm." Their distribution and relative amount correspond rather well to the location and intensity of cytoplasmic basophilia, and digestion of cells in ribonuclease solutions destroys the structures [24]. They appear sometimes as elongated, closed loops with two sides closely apposed, as in the normal pancreatic cell, sometimes as spherical vesicles, as in the syncytial trophoblast of the early human placenta, and sometimes as complex, branched structures, as in rapidly proliferating fibroblasts (Fig. 14). These structures appear to be osmotically closed systems, since they can be caused to dilate by exposure to hypotonic solutions and to shrink in response to high external osmotic pressures [24].

In the pancreas of the starved albino mouse the ergastoplasmic sacs are greatly decreased in number, as are the stored products of secretion, the zymogen granules. Upon refeeding animals with such depleted cells, there is a rapid reformation of the ergastoplasmic membranes which precedes the formation of new zymogen granules. The newly formed ergastoplasm exhibits frequent appearances strongly suggesting that small vesicles are budded off from the larger parent sacs. Within the lumens of these vesicles, a homogeneous substance appears; with time this substance becomes denser, until at last it is indistinguishable from newly formed zymogen granules. These zymogen granules, incidentally, are enclosed by a thin, osmiophilic membrane. It appears, therefore, that the ergastoplasmic sacs and their derivatives represent a structure within which the secretory products of the pancreatic acinar cells are formed and stored [24].

Similar cytological phenomena have been observed in other cells. In the thyroid gland, transition forms may be seen linking the intracellular colloid droplets on the one hand to the flattened ergastoplasmic sacs on the other (Fig. 12). In hypophysectomized rats the number of ergastoplasmic membranes and colloid droplets both are greatly reduced, whereas in glands in activated states, induced either by exposure to cold or by the administration of thiouracil, the number of droplets is greatly increased, and the ergastoplasmic membranes are dilated by the presence within the lumens of a colloid-like substance [6]. Similarly, in the glandular cells of the sow's uterus during pregnancy, ergastoplasmic sacs are associated with vesicular buds, the lumens of which contain varying amounts of a dense homogeneous substance similar to the secretory products of the cell (Fig. 13) [9]. We have alluded to the complex, branching ergastoplasmic sacs of proliferating fibroblasts; in various mammalian placentas we have observed considerable quantities of homogeneous material within the lumens of the sacs in cells located in the fetal connective tissues (Fig. 14). This appearance is associated presumably with synthetic activity exhibited by the rapidly growing tissues.

MITOCHONDRIA. Mitochondria are recognizable in electron micrographs by their size, shape, distribution, density, and characteristic internal structure of folded membranes. These internal folds have been called "cristae" by Palade [19]. They are lamellar in most mitochondria, but in a few locations they appear circular in cross section and so are presumably villous.

Mitochondria are exceedingly pleomorphic. They vary greatly in size, shape, density, and complexity of their internal structure. For example, mitochondria in the proximal convoluted tubules of the kidney are elongated, filamentous structures with complex internal folds, whereas those in lymphocytes are sparse, small, oval bodies with a dense matrix and only a few simple internal folds. They sometimes differ in different parts of the same cell. Those in the perikaryon of neurons may differ in size, density, and complexity from those located in the dendrites or in the axon of the same cell. They alter their appearance in different stages of the cell's existence. In unfertilized rabbit eggs they are few in number and contain a dense matrix and a simple internal structure. By contrast, cells of the developing blastocyst have many bizarrely shaped mitochondria the matrix of which is relatively lucent and the internal structure of which is complex. Moribund cells, frequently encountered in prismatic epithelium such as the vitelline endoderm, have mitochondria greatly altered as compared with neighboring, healthy cells. The mitochondria in the yolk-sac epithelium of the guinea pig at term are swollen, vacuolated, and degenerate in appearance as compared with the same organelles earlier in gestation [5]. They exhibit alterations in size, shape, and number in pathological transformations of liver cells [13]. In starvation, the mitochondria become altered and sparse in the liver [11] and practically disappear from the pancreas [2, 24]. In both organs subsequent refeeding is followed by rapid restoration of these organelles.

Mitochondria appear also to be involved in the formation or segregation of lipide inclusions within cells [14]. In the outer zones of the adrenal cortex, lipide droplets characteristically occur profusely. In rats subjected to stress these droplets become scarce; following removal of the stress, they are restored to their original size and number. These cells contain many mitochondria which are large, turgid, and closely packed together. Their outer walls are composed of double membranes; internally, numerous villus-like folds may be seen which are frequently circular in cross section. The density of the mitochondrial matrix varies greatly, some being quite lucent whereas others contain a substance so osmiophilic as to obscure partially the details of the internal folds. The lipide droplets, recognizable by their extreme osmiophilia and consequent density, can occasionally, in very thin sections, be seen to have limiting outer membranes and faint internal structures similar to the internal folds of mitochondria. Thus a transitional series can be traced from unequivocal mitochondria through mitochondria containing a strongly osmiophilic matrix to frank lipide droplets.

In the deeper zones of the adrenal cortex lipide droplets are sparse and irregular in size and frequently occur in cells containing pycnotic nuclei and exhibiting signs of exhaustion or senescence. These appearances have been interpreted as degenerative by light-microscopists. Within these moribund cells occur fewer mitochondria than in the larger, more turgid cells of the outer layers. From these facts one may speculate that mitochondria are transformed into lipide inclusions during the secretory life of the cell and that they are used up in the process. The older, senescent cell might, therefore, be regarded as having been exhausted of its normal complement of mitochondria.

The sequence of transitional forms relating mitochondria to lipide droplets is not confined exclusively to cells of the adrenal cortex. Similar sequences have been seen in cells from the corpus luteum [15].

Mitochondria have long been known to be sensitive to alterations in the physical environment of cells. Anoxia, altered pH, and other factors frequently cause a swelling and ensphérulation of mitochondria. In electron micrographs swollen forms are often encountered and are ordinarily ascribed to faulty procedures of fixation. Palade [18] and Porter and Kallman [22] counsel electron microscopists to remove tissue rapidly, else the anoxia beginning with the death of the animal causes mitochondrial swelling and distortion. Weiss [24] demonstrated that immersion of tissue in hypotonic solutions caused mitochondria to swell, whereas hypertonic media caused shrinkage. Sjöstrand and Rhodin [23] studied the conditions of fixation, particularly of pH, which permitted optimal preservation of mitochondria from renal tubules. Nevertheless, swollen mitochondria cannot, alone, be accepted as evidence of poor fixation, since two adjacent cells sometimes exhibit mitochondria of different sizes, thus indicating that the physiological status of the cell also has some influence.

From the foregoing account it appears that mitochondria differ in appearance when the organelles from different tissues are compared, when those from the same tissue are examined at different phases of their functional cycles, and when *post mortem* or *in vitro* changes occur in the cell's environment. Mitochondrial alterations can also be induced by the introduction, *in vivo,* of toxic agents. Dempsey and Wislocki [7, 8] studied the deposition of silver in the tissues of rats, mice, and guinea pigs after chronically administering dilute solutions of silver nitrate as drinking water. In such animals, dense, granular aggregates appeared in the tissues and were located for the most part in the basement membranes associated with epithelia and small blood vessels, and in intracellular deposits within macrophages. Occasional granules were also detected in parenchymatous cells. In the liver and pancreas, and in certain glial cells, these intracellular deposits were aggregated within vacuoles delimited by a membrane from the surrounding cytoplasm. These vacuoles resembled mitochondria in size, shape, and distribution. In those containing only a few granules of silver, bilaminar walls and internal membranes were evident, and the amorphous matrix was similar in density to that of mitochondria (Figs. 15, 16).

Similar observations have been made by Weiss [25] on mitochondrial damage induced by the *intra vitam* administration of neutral red. As observed in surviving pancreatic cells with the light microscope, this dye is segregated as neutral red bodies. These bodies may be related to mitochondria by transitional forms in which the characteristic, but distorted, internal structure of mitochondria is evident.

3. Summary

The present armamentarium of histochemical methods permits observations on the changes in composition of cellular structures which take place with alteration of the activity of cells. Microchemical analyses provide greater specificity and quantitative accuracy than do histochemical procedures, the principal virtue of which is their spatial or morphological precision. Used in conjunction with each other, the two kinds of cytochemistry constitute methods for investigating the chemical composition of cellular components, knowledge of which is necessary for understanding the cell's functional capacities.

The morphological characteristics of structures too small for resolution with visible light have been studied previously by indirect methods, chiefly polarization optics and X-ray diffraction. Recently electron microscopy and its ancillary procedures for fixation and sectioning have allowed direct observation of components as small as 20–30 A. With these methods, the plasma membranes of cells have been found to be more complex and diverse than previously known. Mitochondria have been seen to possess a characteristic internal structure which differs in the cells of different tissues and which can be modified experimentally. The ergastoplasm, a basophilic component of cells, has been found to have characteristic morphological appearances and to undergo alterations when the function of the cell is modified experimentally.

Information derived from the current histochemical and electron microscopical procedures point toward a compartmentalization of cellular functions. With these methods it should prove feasible to make considerable advances in our knowledge concerning cellular morphology, cellular chemistry, and cellular physiology.

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3. *Structural Specializations of the Cell Surface*[1]

BY DON W. FAWCETT

KNOWLEDGE OF THE STRUCTURE, physical properties, and behavior of the cell surface is of fundamental importance to an understanding of absorption, secretion, fluid transport, and other histophysiological processes. Cytologists have made valuable observations bearing upon all of these, but their contributions have been greatly limited by the fact that, with the ordinary microscope, it is possible to visualize the structure of only a few of the most conspicuous specializations of cell surfaces. The interpretation of these was often highly controversial because important details of their structure were at or beyond the limits of resolution of the optical microscope. The development of the electron microscope has extended the visible range almost a hundredfold and has opened up for the cytologist a vast and relatively unexplored area of cellular fine structure of dimensions lying between 20 A and 2,000 A. With this instrument and other modern methods of structural analysis, it may now be possible to close many of the gaps in our knowledge of the cell surface which have retarded progress in cell physiology.

The present paper does not attempt a complete coverage of the whole broad subject of the cell surface but presents a brief account of certain specializations of the free and the contact surfaces of cells whose finer structure has recently been clarified by electron microscopy.

1. Specializations of the Free Surface

STRIATED BORDER. Over a century ago the specialized border of intestinal epithelium was depicted by Henle [30] as a homogeneous, refractile layer, devoid of visible internal structure, and this was regarded as an inert, protective coating or cuticle elaborated by the cell

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(Fig. 18). At a later date, delicate vertical striations were recognized within it, and these were interpreted by some as minute pores or canaliculi penetrating the substance of the cuticle and providing pathways for the absorption of nutrients [23, 32, 2]. Others attributed this striated appearance to the presence of slender rods embedded in an amorphous matrix [12]. Since the structures in question were very near the limit of resolution of the light microscope, it is not surprising that the "pore" vs. "rod" controversy continued from 1850 until 1950, when the first electron micrographs of sections of the intestinal border were published by Granger and Baker [26] and by Dalton, *et al.* [13]. These studies clearly settled the dispute by revealing the presence of closely packed, rodlike processes on the cell surface.

These slender processes, now commonly called *microvilli,* are 80 to 100 mμ in diameter and 0.6 to 0.8 μ in length (Figs. 19, 20). They are enclosed in an extension of the plasma membrane and have a fine-grained protoplasmic content which is continuous below with the cytoplasm at the apex of the cell. The protoplasm in the interior of the microvilli is relatively dense, and this denser material sometimes extends downward from their base for a short distance into the apical zone of cytoplasm (Fig. 20). The resulting vertical streaks are simply continuations of the denser content of the villi into the less dense cytoplasm of the cell body and although they have been referred to as "rootlets" [8], they are in no way comparable to the fibrous rootlets of cilia. Just below the lower margin of the striated border they blend into a poorly defined dark band which extends transversely across the end of the cell. There is reason to believe that these denser streaks vary in prominence in different nutritional conditions. It is likely that they are responsible for earlier reports of a row of basal granules at the proximal ends of the "rods" of the striated border [12].

A clear zone is visible with the light microscope immediately beneath the specialized border. In cytological preparations mitochondria are found to be absent from this paler zone, although they are very abundant in the subjacent region of the cytoplasm. W. J. Schmidt, in an investigation of intestinal epithelium with the polarizing microscope, demonstrated birefringence in the striated border and in the cell body which was positive in relation to the long axis of the cell, but in the region corresponding to the *clear zone* of ordinary microscopy there was a transverse band with a negative sign of birefringence [48]. It was inferred from these observations that this boundary zone between the striated border and the cell body possessed a foliate structure in which fibrous proteins ran in a plane parallel to the free surface of the epithelium. The electron microscope now provides a direct confirmation of these conclusions. When intestinal cells of some species are examined at high magnification, delicate filaments about 50 A in thickness are abundant in this region, and although their orientation is variable, their prevailing direction is parallel to the surface. Fine fibers of this kind are seen in intestinal epithelium of mammals, but they are more conspicuous in lower forms, such as *Lumbricus* and *Elliptio* where the epithelium of the gut is provided with both microvilli and motile cilia. These filaments no doubt form the fibrous framework of a firmly gelated ectoplasmic layer which supports the erect microvilli of the striated border and provides a firm implantation for the basal bodies of the cilia when these are present.

According to older concepts of the structure of the striated border, the "rods" (*Stäbchen*) were embedded in an amorphous matrix, but current accounts of its structure, based upon electron microscopy, do not mention a matrix surrounding the microvilli. The positive periodic-acid Schiff staining reaction of the border is attributed to the presence of neutral mucopolysaccharides within the cell processes instead of between them [37, 34]. Although it is the consensus at present that the striated border is made up of free cell processes, the possibility cannot be denied that in life the interstices between the processes may be occupied by a polysaccharide product of the cell which is not preserved by osmic acid fixation.

The microvilli of the intestinal border are commonly regarded as a device for increasing the effective surface for absorption, and it has been estimated by Granger and Baker [26] that a single cell may have 3,000 of such processes and a square millimeter of intestinal mucosa as many as 200,000,000. There can be no doubt that they do provide a great amplification of surface, but their relation to absorption seems less certain than formerly since the electron microscope has revealed similar processes on a large variety of epithelia, some of which have no known absorptive function, others of which are definitely secretory. Thus a sparse covering of microvilli is found on the mucous neck cells, parietal cells, and peptic cells of the stomach; epithelium of the gall bladder and choroid plexus [14, 15]; various ciliated epithelia [22]; epithelium of the yolk sac [18]; the uterine epithelium [19]; the mesothelial cells of serous membranes [38]; and the surfaces of the sinusoids and bile capillaries of hepatic cells [21] and the Malpighian tubules of insects [3]. Among these, the cells of the choroid plexus and the parietal and mucous cells of the gastric mucosa certainly are secretory. Thus the presence of microvilli on epithelial cells in general cannot be taken as unquestionable evidence of absorptive function. The significance of such processes on secretory cells is not known.

BRUSH BORDER. Histologically the brush border of the nephron closely resembles the striated border of the intestine (Fig. 17) and historically a similar disagreement has existed over the structural basis of its vertical striations; some workers attribute this appearance to pores and others explain it on the basis of rods. This controversy was not immediately resolved by the electron microscope. On the one hand, Gautier and Bernhard [24] reported that the border was made up of complex branching lamellae that joined to form the walls of vertically oriented pores or tubules. On the other hand, Pease and Baker [39] and Dalton, *et al.* [13] considered it to be comprised of slender rodlike processes. Sjöstrand and Rhodin [51] at first described a border of "ducts" or "tubules" closed at both ends, but they later abandoned this interpretation in favor of one of closely approximated cell processes closed at their tip but in open communication at their base with the subjacent cytoplasm [47]. It is now generally accepted that the brush border in the kidney is made up of processes (microvilli) rather than tubules.

Attention has recently been directed to certain differences between the specialized border of the proximal segment of the nephron and that of the intestine. The border processes on the intestine appear to be independent of one another (Fig. 22), whereas those in the kidney often seem to be embedded in a relatively dense amorphous matrix (Fig. 23). Horizontal sections through the border thus have a honeycomb pattern in which the cross sections of the microvilli appear as sharply defined circular areas of low density, outlined by an extracellular substance of greater density (Fig. 23). This appearance is probably the basis for the earlier descriptions of tubules or ducts. In some places V-shaped clefts occur in the border between tufts of microvilli that are held together by a matrix. In other instances the matrix is entirely lacking, and the border seems to be made up of free processes. These different appearances are sometimes noted in neighboring areas of the same preparation. It is considered most likely that they are due to local variations in the quality of fixation, but the possibility cannot be excluded that they reflect true differences which exist *in vivo*. The amorphous substance of the border is of about the same density in electron micrographs as the homogeneous component of the basement membrane. Both give a positive reaction with the periodic-acid Schiff reaction (Fig. 17), and it is suggested that both may be a mucopolysaccharide product of the epithelial cells. It is probable that a matrix of this kind would be no barrier to diffusion and would not invalidate the current concept of the brush border as a device for increasing the area of the cell surface exposed to the glomerular filtrate.

Electron micrographs have made it possible to make measurements upon which to base an estimate of the magnitude of the increase in surface afforded by the brush border. Policard and his co-workers [43] found that the individual microvilli in the rat nephron were 3μ long, and 0.5μ in diameter and they numbered about 128 per square micron of brush border. Using these measurements and the mean values for the circumference of the lumen and the length of the proximal segment, they estimated that the total surface of all the border processes in a nephron would be about 26 square millimeters. If there are 2 to 2.5 million nephrons in the two kidneys of man, as has been claimed, then the total surface of the brush border processes would amount to as much as 50 to 60 square meters. The existence of such an enormous absorbing area goes a long way toward accounting for the extraordinary capacity of the nephron for concentration of the glomerular filtrate.

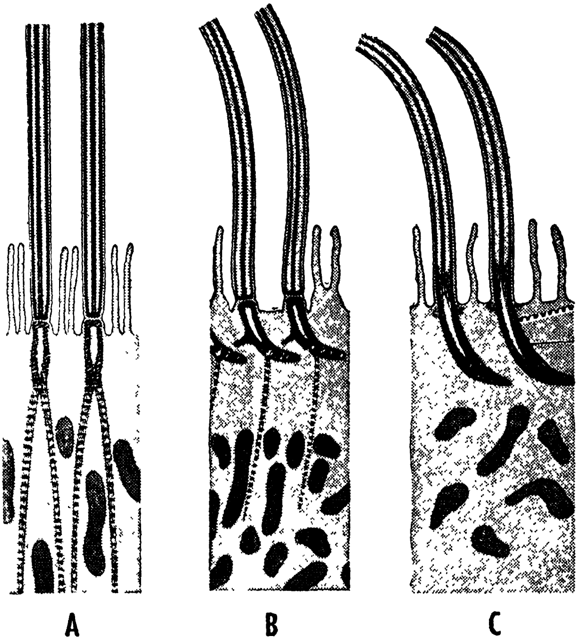
Another feature of the brush border of the renal epithelium which distinguishes it from the intestinal striated border is the presence of many slender tubular invaginations of the cell membrane that arise from the depths of the interstices between the microvilli and run downward into the apical zone of cytoplasm (Fig. 21). These run a tortuous course and are closely associated with clear vacuoles, which are common in this part of the cell. It is thought that the vacuoles may arise as expansions of the tips of the intracellular tubules and that the latter may be pathways through which considerable quantities of fluid are taken into the cell by a process akin to the pinocytosis observed in various kinds of living cells in tissue culture.

Similar intracellular tubules or canaliculi have been described in association with the specialized border of the yolk sac epithelium of the guinea pig placenta, which is also a site of rapid absorption. Dempsey [18] has offered the interesting speculation that readily diffusible substances may enter these cells through the microvilli, whereas proteins and other large molecules may gain access to the interior of the cell through this extensive system of tubular invaginations of the surface.

CILIA. Under the light microscope cilia are generally optically homogeneous, but with special staining methods an *axial filament* can be made out running longitudinally through the entire length of the cilium. The cilia take origin from a row of dense *basal corpuscles* situated in the cytoplasm just beneath the free surface and usually emerge from the cell through a refractile *striated border*. In invertebrates *fibrous rootlets* can be seen arising from the lower pole of each basal corpuscle and extending downward into the cytoplasm. These may converge to form a conspicuous *cone of rootlets* reaching to the level of the nucleus or beyond (Fig. 24). In mammals, ciliary rootlets are poorly developed, and in many epithelia they appear to be absent.

The birefringence of cilia and the observation that they sometimes fray at the tip into a tuft of finer fibrils [27] suggested to light microscopists that the so-called axial filament was actually a compound structure made of multiple individual strands. This was borne out by the early electron microscope studies which revealed the presence of several longitudinal filaments [49, 31]. The number was estimated by various workers to be between nine and twelve. After a careful examination of cilia from a considerable number of different plant forms, Manton [35, 36] reported that the number of filaments was always eleven, of which nine appeared to be double strands while the remaining pair were single and seemed to differ slightly from the others. From these studies on dissociated filaments it was postulated that in the intact cilium the odd pair was centrally situated in the bundle and the nine double strands were disposed around the periphery. When epithelial cilia of several animal species were examined in thin cross sections [22], the number eleven proved to be constant and the arrangement predicted by Manton was found to obtain: two single filaments in the center and nine double filaments in a circle around them (Fig. 25). The entire bundle of filaments is embedded in a protoplasmic matrix and enclosed in a ciliary membrane which is continuous with the plasma membrane of the supporting cell. The filaments in cross section appear tubular rather than solid because their surface is more dense or binds more osmium than their interior (Fig. 25). The central pairs of filaments are similarly oriented in all of the cilia on a given cell and are thought to lie in planes perpendicular to the direction of ciliary movement. These longitudinal filaments are assumed to be contractile, but the electron micrographs provide no clue to the mechanism by which the asynchronous contraction of the several filaments is controlled to bring about the characteristic pendular motion of the cilia.

The foregoing description of the internal structure of the cilium has been found to apply to protozoa (*Paramecium*) [50]; bryozoa (*Pectinatella*); annelida (*Tubifex* and *Lumbricus*); mollusca (*Elliptio, Ensis, Mya*); amphibia (*Rana, Triturus, Ambystoma*), and mammals (*Mus, Rattus, Homo*). Although minor variations and exceptions may yet be found, it seems safe to state that motile cilia throughout the animal kingdom have the same basic structure. The phylogenetic or adaptive significance of this particular internal organization remains to be explained.



TEXT FIGURE A. Diagrammatic representation of the structure of the ciliary apparatus of (A) a mollusc (*Elliptio*), (B) an amphibian (*Rana*), and (C) a mammal (*Mus*). The cross-striated rootlets are best developed in the invertebrates, smaller in amphibia, and rudimentary or absent in mammals. The longitudinal filaments of the cilia in the mammal appear to be continuous with the substance of the basal corpuscle. (From Fawcett, *The Laryngoscope*, *64*, 557, 1954.)

The intracellular portion of the ciliary apparatus, on the other hand, varies considerably in different species with respect to the shape of the basal corpuscles and the degree of development of the associated fibrous rootlets. The basal corpuscles are arranged in precise rows and each has a dense cortex and an interior of very low density which gives it the appearance of having a central cavity (Fig. 26). In some animal species the longitudinal filaments of the cilia are continuous with the dense cortical layer of the basal corpuscle, but in others they terminate in a horizontal base plate which is distinct from the basal corpuscle but attached to its flat upper surface. The intracellular rootlets which arise from the basal corpuscle are cross-striated, and a suggestion of cross-striation is often detectable in the lower part of the basal body (Text Fig. A).

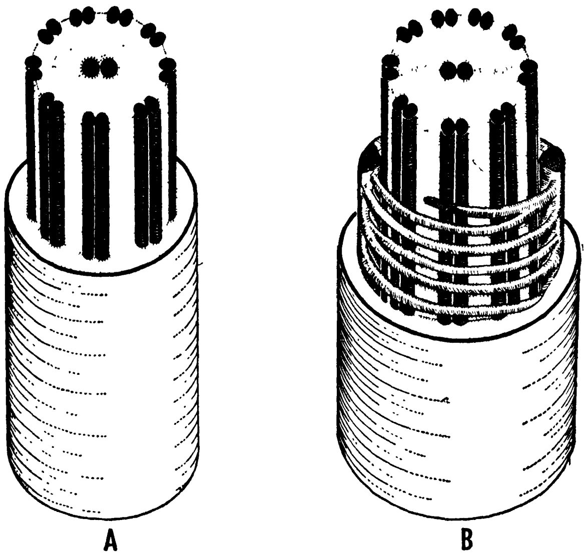
In a previous account of these rootlets [22] they were described as having a conspicuous cross-banding at regular intervals of 600 to 700 A along their length, with one or possibly two narrower bands discernible within the major period (Fig. 28). Electron micrographs of higher resolution recently obtained on the ciliary rootlets of a mollusc (*Elliptio*), an earthworm (*Lumbricus*), and a bryozoan (*Pectinatella*) indicate a more complex cross-banded structure than that previously described. The fine structure of the rootlets seems to be much the same in the three species, but by far the clearest pictures were obtained in *Lumbricus,* where it was apparent that there are five bands in each period instead of two (Fig. 27). In this material the broad band, previously noted, can now be resolved into three closely spaced narrow bands of about the same density, and in addition to the narrow dense intraperiod band seen before, there is at least one other of lower density which hitherto escaped detection.

It is still a moot question whether ciliary rootlets are simply anchoring structures or whether they play an active part in the movement of the cilia. In *Pectinatella* at least three rootlets arise from each basal body. Two of these are disposed horizontally and the third is vertical (Fig. 26). If these rootlets were contractile, their shortening in a particular sequence could impart a pivotal movement to the basal corpuscle which would be transmitted to the cilium. In *Elliptio* and in *Mya* two rootlets take origin from each basal corpuscle and diverge at an acute angle as they run down into the cell body (Fig. 28). In *Rana* a single, short, vertical rootlet is the rule, and it is difficult to see how this could contribute to the vibratile movements of the cilium. In mammals the rootlets are rudimentary or absent, and thus, whatever their function may be, it is obvious that they are not essential for ciliary beating.

A repeating period of about 640 A and a five-banded intraperiod structure have come to be considered identifying characteristics of collagen. The production of collagen precursors is attributed exclusively to cells of mesenchymal origin and actual fibrogenesis is believed to take place only extracellularly. Therefore, it would be most unexpected to find collagen within epithelial cells. If the rootlet fibers are collagen (and we believe they are not), then in the light of their 660–700 A period and their five-banded intraperiod structure, we must revise our ideas concerning the value of the 640 A period for the visual identification of collagen. Further clarification of this problem awaits the isolation and chemical characterization of these fibers.

FLAGELLA. Whereas cilia usually execute simple pendular movements, flagella beat with an undulatory motion in two or three dimensions. If the type of movement were determined by the number and disposition of the internal filaments, one might expect these two types of motile cell process to differ considerably in their internal organization, but quite to the contrary the protozoan and epithelial flagella that have been studied to date are almost identical to cilia in their fine structure (Text Fig. B).

Several earlier electron microscope investigations were on sperm flagella examined directly or after dissociation by ultrasonics, and these led to erroneous reports of eight, nine, or twelve filaments [28, 45]. More recent studies carried out on thin sections of amphibian and mammalian spermatozoa reveal that the number of filaments is always eleven in the principal piece of the sperm tail, and these are arranged exactly as in cilia [20]. The sperm flagella of mammals do, however, have an additional component not found in cilia. This is a circumferential filamentous wrapping which on superficial examination appears to have a spiral course around the longitudinal fiber bundle and hence has been called the *cortical helix* [7, 5, 6]. Close examination of this structure in sections of testis indicates that Randall's [45] concept of a double helix with the turns making an angle of 85° with the long axis of the flagellum is altogether too mechanical. Instead of being comprised of a pair of continuous, independent filaments wound spirally around the cylindrical fiber bundle, the dense strands of the wrapping branch and anastomose (Figs. 31 and 32) and the successive turns are held together by their attachment to two longitudinal bands which run on opposite sides of the flagellum in the plane of the two central filaments (Fig. 33). While it can be argued that the pictures obtained in the testis represent formative stages in the development of the true helix described by others in ejaculated spermatozoa, it seems more plausible that the higher resolution electron micrographs permitted by thin sections have disclosed for the first time the actual structure of this wrapping which is interposed between the longitudinal filaments and the flagellar membrane. It appears to be characteristic of mammalian spermatozoa. In the amphibia studied it is absent, but a substance of similar density forms a broad fin along one side of the flagellum (Fig. 29). A thin sheet of this dense material projects outward from the shaft of the flagellum and has in its free edge a thickening which in *Bufo* is oval or elliptical in cross section (Fig. 29) and in *Triturus* is trifoliate (Fig. 30). This entire fin is enclosed in an extension of the flagellar membrane that is closely applied to its surface. The base of the fin is always in the same plane as the central pair of longitudinal filaments. This would seem to



TEXT FIGURE B. Diagrammatic representation of the structure of a cilium (A) and of the principal piece of the mammalian sperm flagellum (B). In the sperm flagellum the longitudinal bundle of filaments is encircled by a wrapping of dense strands which branch and rejoin and are held together by their common attachment to two longitudinal strands of the same material that run along opposite sides of the flagellum in the same plane as the central pair of filaments. (From Fawcett, *The Laryngoscope, 65,* 557, 1954.)

provide additional evidence for the statement made earlier with respect to cilia, that the plane through the two central filaments is normal to the direction of beating. In these sperm flagella, if the fin is to be effective in propulsion it should be perpendicular to the plane of flagellar movement. The character of the movements of sperm flagella is a matter of dispute, but the structure of those described here would seem to be more consistent with a two-dimensional undulation of the flagellum than with a spiral wave motion. The function of the thickening along the edge of the fin is unknown, but one may speculate that it provides some degree of stiffness or that it serves as a weighted keel to counterbalance torque and stabilize the spermatozoan during its swimming movements.

2. *Specializations of the Contact Surfaces of Adjacent Cells*

Since Recklinghausen's studies in 1862 on silver impregnations of squamous epithelia, the cohesion of cells has generally been attributed to a viscous *intercellular cement.* Specializations such as *desmosomes* and *terminal bars* have been thought of as playing a subsidiary role in the maintenance of cell attachment in certain epithelia. The results of electron microscopy suggest the need for a re-evaluation of the respective roles of these elements in cell cohesion.

INTERCELLULAR CEMENT. In electron micrographs the boundaries between cells appear as parallel osmiophilic lines 80–100 A in thickness and 150–200 A apart (Figs. 34, 36, 40, 43). Each of the dense lines apparently represents the full thickness of the lipoprotein plasma membrane of one of the cells, and the two are separated by an intercellular cleft of very constant width. Just what occupies this narrow interspace is not at all clear. In most electron micrographs it appears empty, but in occasional preparations there is seen between the membranes a homogeneous substance of very low density which could correspond to the postulated intercellular cement. With the light microscope a delicate line of material reactive with the periodic-acid Schiff stain is sometimes visible at the junction between cells (Fig. 17). It is not known whether this staining resides in unsaturated groups of a lipide component in the confronted cell membranes or whether it is due to a very small amount of a mucoprotein substance between them. The existence of an intercellular "cementing" substance cannot be ruled out on the basis of present evidence, but whether it could be effective in such an incredibly thin layer seems doubtful. The closeness of the plasma membranes of neighboring cells as seen with the electron microscope suggests that perhaps cell cohesion should be thought of in terms of long-range forces of attraction between molecules rather than in terms of an intervening layer of adhesive. In accord with this concept there is an accumulating body of experimental evidence indicating that the selective cohesion between cells of the same type is mediated by surface forces similar to those responsible for the interaction of antigen and antibody [53, 54]. It is well known that lymphocytes have no difficulty in loosening the bond between neighboring endothelial or epithelial cells as they migrate through into the lumen; and as the lymphocyte passes, the intimate relation of the cell surfaces is immediately reestablished. This is easier to comprehend as a temporary separation and reunion of sites of specific attraction on the respective surfaces than as a dissolution and immediate reformation of intercellular cement.

DESMOSOMES. Desmosomes have been described on a number of epithelial cell types but are seen in their most typical form in the stratum spinosum of stratified squamous epithelium. Here the cells are joined by many protoplasmic processes which traditionally have been called *intercellular bridges*. These traverse narrow intercellular clefts that are believed to be filled with some sort of ground substance [57]. The *desmosomes* (granules of Ranvier, nodes of Bizzozero) show up under the light microscope as darkly stained bodies at the midpoints of the intercellular bridges. They give a positive staining reaction with Sudan black and with the acid hematein test for phospholipides [56, 57]. In stained preparations and in fresh material examined by phase contrast, delicate *tonofibrillae* can be followed from the cytoplasm of the cells into the bridges, where they seem to converge upon the desmosome, but it cannot be determined by light microscopy whether they end there or pass through from cell to cell.

Owing to inadequate methods of fixation and thin sectioning, the early electron microscope studies of skin [1, 40] did not resolve the question of protoplasmic continuity through the intercellular bridge and failed to define the nature of the desmosomes. Using improved techniques of specimen preparation, Porter [44] has shown in a recent study of amphibian epidermis that there is no actual continuity between the cells. Instead of having intercellular spaces between cell processes, the confronted cell surfaces in this amphibian material are relatively straight and closely coherent throughout (Fig. 34). The desmosomes consist of regularly spaced, local thickenings of the opposing cell membranes, from which tufts of fine filaments radiate toward the interior of the cells (Fig. 34). The filaments run right up to the membranes from both sides and seem to end in them, for where the cells have been pulled apart in preparation, there is little or no indication of filaments passing through from one cell to the other. Filaments of the same sort, organized in loose skeins, run a serpentine course in the cytoplasm of the cells, and it is thought that they may be keratin.

Although there is nothing in the fine structure of the desmosomes to indicate how they might serve to maintain firmer cohesion of cells, there is reason to believe that this is their function. In certain stratified epithelia where intercellular bridges have been described with the light microscope, the electron microscope reveals a close co-aptation of cell surfaces that are studded with numerous desmosomes. In these epithelia the common appearance of intercellular bridges is thought to result from cell shrinkage, with persisting adhesion at the desmosomes.

Although desmosomes are seldom seen with the light microscope except in stratified squamous epithelia, they are found with the electron microscope irregularly distributed over the contact surfaces of the cells in many columnar and cuboidal epithelia. They have been observed on the epithelium of the intestine, uterus, kidney, oviduct, and parietal layer of Bowman's capsule, in the follicular cells of the amphibian testis, and in a renal cell carcinoma (Figs. 36, 37). In view of the widespread occurrence of desmosomes, it is not surprising to discover on a search of the literature that intercellular bridges have been described in a wide variety of epithelia. These include the serous membranes [46], the outer root sheath of hair [9]; intestinal and gastric epithelium [11]; epithelium of Descemet's membrane [52]; kidney, thyroid, uterus, seminal vesicles; salivary glands; and stomach [33]. Indeed, Kolossow in 1898 described a special method of preparation which made it possible to demonstrate intercellular bridges in nearly all epithelia. It now seems likely that his fixative caused considerable shrinkage but permitted cell surfaces to remain adherent at the desmosomes, thus producing the appearance of intercellular bridges.

TERMINAL BARS. In vertical sections of certain columnar epithelia stained with iron hematoxylin a series of dark dots are visible at the interfaces between adjacent cells just below their free border. In horizontal sections at this level these structures appear as dense bars or rods which intersect at the corners to form a hexagonal network outlining the cells. These dark-staining lines are called *terminal bars* (*Schlussleisten, bandes de fermeture, bandelettes obturantes*). They were first described by Prenant and later interpreted by Bonnet [4] and by Zimmermann [58] as consisting of a special intercellular cement substance (*Kittleiste*) that served to hold together the edges of the cells and to seal up the interstices so that material is prevented from passing between the cells. Other investigators have regarded them as part of the cell surface, a special form of desmosome [16]. In support of the latter view it is pointed out that in certain columnar epithelia, tonofibrillae converge upon the terminal bars in much the same manner as they do upon the desmosomes of stratified squamous epithelia. Moreover, where cells are forced apart, half of the terminal bar goes with each cell, further attesting to its double nature.

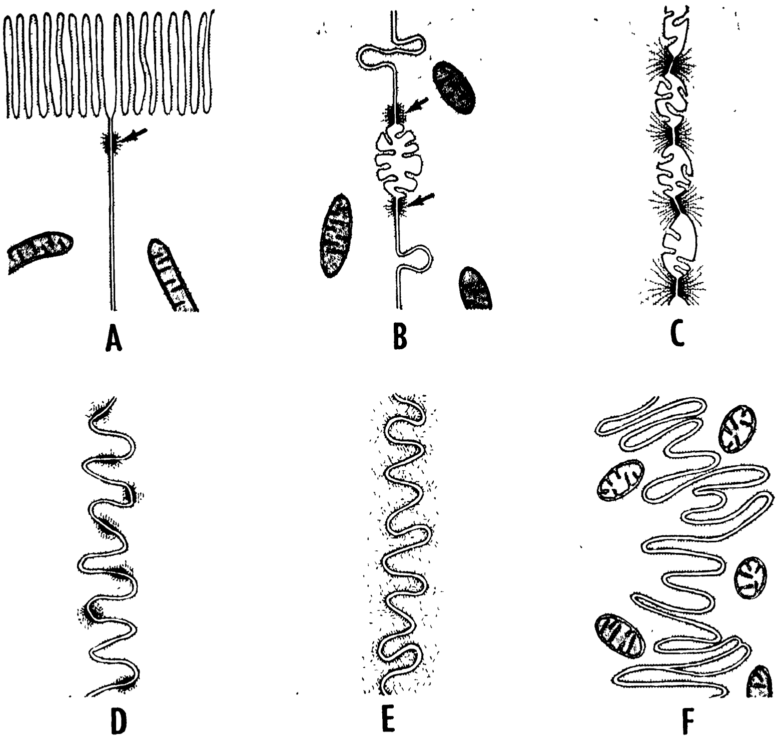
At the sites where terminal bars are observed with the light microscope, the electron microscope discloses opposite thickenings of the plasma membranes of adjacent cells which, in vertical section, are virtually indistinguishable from the desmosomes already described. The tuft of radially oriented filaments, which was a prominent feature of the desmosomes in stratified squamous epithelia, is less conspicuous in the terminal bars of simple epithelia. Nevertheless there is an accumulation of dense cytoplasmic material on the inner aspect of the thickened area of the cell membrane. When seen in horizontal sections passing just beneath the free surface of the cell, the dense material of the terminal bar forms two rather sharply defined dense bands on either side of the membranes of the adjoining cells (Fig. 37). In micrographs of good resolution, delicate filaments can be made out within the dense substance of these bands. The majority of the filaments seem to be parallel to the cell surface, but a few run into the cytoplasm perpendicular to the membrane.

Thus the current concept of terminal bars as local intercellular accumulations of cement substance is erroneous. They are bipartite structures of which the two symmetrical halves are integral parts of the surface of neighboring epithelial cells. They have essentially the same fine structure as desmosomes and in transverse sections are indistinguishable from them. The terminal bars are, however, long bands generally situated near the free surface of the epithelium, whereas the desmosomes are small plaques widely distributed over the contact surfaces of the cells.

INTERDIGITATION OF CONFRONTED CELL SURFACES. Kölliker in 1889 clearly illustrated an interdigitation of corrugated surfaces on cells in the stratum spinosum of the epithelium on the tongue. In later writings and textbooks, however, it has been customary to depict these cells as if they were connected by many intercellular bridges traversing narrow intercellular spaces. As a rule, no important distinction is made between stratified squamous epithelia of the oral cavity and those of the skin, cervix, or vagina with respect to the relations of the cells in the stratum spinosum.

Electron micrographs of the epithelium of the tongue and gingiva disclose that the cells of the stratum spinosum are not provided with "prickles" or "spines" but have on their surfaces alternating grooves and ridges which closely interlock with the ridges and grooves of the neighboring cells, leaving no intercellular spaces (Figs. 38, 39). The desmosomes found at irregular intervals on this corrugated surface do not bear a constant relationship to any particular part of the surface contour but fall sometimes on the apex of a ridge, sometimes on its side or in a groove (Fig. 39).

Many bundles of fine filaments course throughout the cytoplasm of the cells of the stratum germinativum, but in the more superficial layers of the epithelium these are concentrated in a dense feltwork under the plasma membrane, and the interior of the cell is occupied largely by glycogen. This extraordinary peripheral accumulation of dense fibrous material (presumably keratin) forms a tough, leathery shell and endows the surface ridges and grooves with considerable stiffness. Thus when cells are pulled apart in the handling of the specimen prior to fixation, the surface corrugations remain (Figs. 40, 41). In the more superficial layers of the epithelium the desmosomes are lost or obscured by accumulation of keratin, and at the same time the surface contours become flatter and their interdigitation more shallow (Fig. 42). Some vestiges of the ridges persist, however, even on cells desquamated into the oral cavity, and they can be detected with the phase contrast microscope as a fingerprint-like pattern on the surface of the squamous cells. The interlocking, tongue-and-groove relation of the cell surfaces in the deeper layers of the epithelium undoubtedly provides some measure of mechanical resistance to shearing forces which might separate the cells. By the same token, the partial obliteration of the surface contours on the more superficial cells is probably responsible for the relative ease with which these cells desquamate.



TEXT FIGURE C. Diagrammatic representation of several types of specialization found on the surfaces of contact between adjacent cells.

A. On the interface between columnar epithelial cells of the intestine *terminal bars* (at arrow) are frequently seen near the free surface.

B. On the contact surfaces of liver cells, *terminal bars* occur (at arrows) on either side of the bile capillary. Near these are studlike processes which project into concavities in the surface of the adjacent cell.

C. In the stratified squamous epithelium of the rodent vagina the cell surfaces are adherent at *desmosomes* and retracted between, giving rise to the so-called "intercellular bridges" of light microscopy. A continuous system of intercellular spaces exists between bridges. Projecting into these spaces are a few short microvilli.

D. In the stratum spinosum of the tongue adjoining cells have closely fitting corrugated surfaces. Numerous desmosomes are distributed over the irregular surface.

E. The partially cornified cells of the superficial layers of stratified squamous epithelium apparently lack desmosomes but the ridges and grooves of the cell surfaces persist.

F. An extraordinarily elaborate intercrescence of cell surfaces is found in the distal convoluted segment of the frog nephron.

There is now good evidence that the squamous epithelia of the female reproductive tract are quite different from those of the oral cavity. The spaces between intercellular bridges in the cervix and vagina apparently are not shrinkage artifacts but are true intercellular channels which exist in life and vary in their size and surface characteristics in different physiological conditions. In a recent study of the cyclic changes in the guinea pig vagina, Burgos and Wislocki [10] have shown that the cells of the stratum spinosum adhere at the desmosomes of the intercellular bridges, but the cell surfaces between are retracted and form the walls of a labyrinthine system of interfacial canals or spaces. During diestrus these are narrow and their walls relatively smooth, but during estrus they enlarge and the walls are beset with numerous short microvilli which project into the intercellular spaces (Text Fig. C). Stratified squamous epithelium is avascular, and the interspaces between cells have been looked upon, in the past, as channels through which metabolites make their way to and from the outer layers. The observation, in different physiological conditions, of changes in the character of the cell surface that is exposed to these spaces lends strong support to the thesis that they are functionally important pathways in the nutrition and metabolism of the epithelium.

A simpler form of interdigitation of cell surfaces is seen in rat liver. There the cells adhere along the entire length of their contact surfaces, except where the cell membranes diverge to form the walls of the bile capillaries. Terminal bars are found along either side of the bile capillary and, when the cells loosen up in prolonged fasting, they remain joined at the sides of the bile capillary [21]. Short stout processes are often seen near the bile capillary that extend from one cell into a corresponding depression in the surface of the adjoining cell (Figs. 46, 47). These processes are sometimes expanded at the tip, while the concavities into which they fit are constricted around their base. The interlocking relationship which results is reminiscent of a mortise and tenon or dovetail joint (Fig. 46). These projections are not sufficiently numerous to increase significantly the cell surface and at present no better suggestion can be made as to their function than that they may help to hold the cells in close contact in the region of the bile canaliculus.

Complicated cell boundaries were recognized in the mammalian kidney as early as 1874 by Heidenhain [29]. The cells of the first portion of the proximal convoluted tubule are described as possessing fluted lateral surfaces that are highly irregular in outline when seen in horizontal sections. This subject was investigated in detail by Zimmermann [58], Del Rio Hortega [17], and Grafflin and Foote [25] in sections impregnated with silver. No satisfactory explanation was offered for the observed intercrescence of the neighboring cells, but inasmuch as it seemed to end with the first half of the proximal convoluted tubule it was assumed to be related to the particular function of that segment. It is now apparent from electron micrographs that the degree of surface complexity actually occurring in the proximal part of the nephron was never fully appreciated with the light microscope. Furthermore, the assumption that irregular cell outlines are confined to that segment is not borne out. Indeed, in the frog the most intricate cell shapes are found in the distal tubule. There the adjacent cell surfaces are thrown up into a great number of folds of varying size. Although the serpentine boundary between cells is extraordinarily elaborate, the two cell membranes remain strictly parallel and a constant distance apart (Text Fig. C and Fig. 43). The regular corrugations on the squamous cells of the tongue seemed to have a rather stable configuration, but the patterns of folds or flutings on the sides of the kidney cells show unlimited variation and it is likely that, *in vivo,* the surface contours are continually changing.

The significance of this elaboration of the lateral cell surface is quite obscure. Cell boundaries which are almost as complex are found in certain parts of the placenta, where the cells also have an absorptive function, and this might suggest that the amplification of lateral cell surfaces is related in some manner to cellular transport, but no metabolic interchange is known to take place between adjoining cells in the nephron that would be promoted by an extension of their interface. Moreover, since the renal tubule is not subject to distention, it is unlikely that such an elaborate intercrescence of the surfaces is needed to maintain cell cohesion. Certainly no satisfactory explanation can be given at present, but future experimental studies may cast more light upon this puzzling specialization of the cell surface.

3. *Specialization at the Cell Base*

Surface complexity in the distal convoluted tubule of the kidney is not confined to the lateral surfaces just described but is in fact even more striking at the cell base. The cells rest upon a fairly thick layer of amorphous material comprising part of the basement membrane (Fig. 45). The plasma membrane is closely applied to this homogeneous layer, but it can be seen to turn inward at many points to form narrow folds or septa. These consist of pairs of parallel membranes about 150–200 A apart, that run far up into the cytoplasm, often extending more than halfway to the free surface of the epithelium. The branching and joining of these membranous septa subdivide the basal part of the cell into a labyrinth of narrow compartments bounded by double membranes on all sides but opening above into the main body of the cytoplasm [47]. The mitochondria, which are concentrated in the lower half of the cell, are lodged in these narrow compartments, and this may account for their vertical orientation as seen with the light microscope.

Horizontal sections through the basal part of the cell (Fig. 44) reveal a bewildering array of double membranes that run a meandering course among the mitochondria, bifurcating, coalescing, and sometimes appearing to end blindly. These complicated septa are prolongations of the surface membrane of the cell, and in consequence of this compartmentation of the basal cytoplasm a very large area of plasma membrane is brought into close relation with a great number of mitochondria situated deep within the cytoplasm. At present, the significance of this relation is purely a matter of speculation, but it is not unreasonable to believe that certain membrane-associated enzymes involved in the transport mechanisms of the cells are thus brought into close proximity with a source of energy in the oxidative enzyme systems of the mitochondria.

Another indication that this intimate relationship of mitochondria to the cell surface is meaningful in relation to absorption and transport is found in the Malpighian tubules of insects. There Beams, *et al.* [3] found attenuated mitochondria actually within the slender microvilli projecting from the free border. This could be thought of as the converse of the situation under discussion here, where the cell surface is brought into relation with the mitochondria by infolding of the plasma membrane. One problem that arises with this interpretation is that the membranes in the frog kidney are so close that even though the space between them does theoretically communicate with the extracellular fluid compartment, it is highly questionable whether movement of fluid or diffusion of molecules could take place over the distances required. In other words, it is difficult to think of this infolding of the plasma membrane as accomplishing an increase in available cell surface. Future experimental studies may show that the septa open up and their membranes become more widely separated under conditions demanding rapid transport, but to date there is little evidence for this.

Modifications of the cell base that are similar to these, but less extensive, have been found in a number of other sites, namely the proximal convoluted tubule of the mammalian kidney [47, 41], the intestinal epithelium of the mollusc *Elliptio* [22], the intestinal epithelium of *Ascaris* [8], the epithelial cells of the chorionic areolae of the pig's placenta [19], the epithelium of the ciliary body of the eye, the pigment epithelium of the retina, and the rodded epithelium of the ducts of various exocrine glands [42]. It is of interest that several of these epithelia are known to have an absorptive function.

4. Summary

With the light microscope only such prominent surface specializations of cells as brush-borders, striated borders, and ciliated borders are visible. The electron microscope has now revealed remarkable order and constancy in the internal organization of these familiar surface modifications and, in addition, has disclosed an unexpected variety of elaborations of the free surface and cell base and of the contact surfaces between cells. While the function of cilia is well understood, the significance of the other surface differentiations is less apparent. Their appearance in electron micrographs suggests that some are specializations for maintaining cell cohesion and others are somehow concerned with absorption, secretion, or fluid transport. These interpretations, however, are speculative, and they will remain so until put on a firmer basis by further work in analytical cytology correlating changes in the configuration of the surfaces with experimental alteration in the physiological activities or environmental conditions of the cells.

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4. Chromosomes:

Their Constitution and Function

BY A. R. T. DENUES

ALTHOUGH THE DISCOVERY of the cell takes us back but one and a quarter centuries, Virchow recognized a century ago the role of the nucleus in the maintenance and multiplication of cells; the fine structure of chromosomes and the particulate qualities of hereditary phenomena had been developed seventy-five years ago by Baranetsky, Roux, and others; the fruitful union in cytogenetics dates but fifty years from Sutton's observations of the strict parallel between genetic and chromosomal behaviors; detailed relations of genes and chromosomal loci became clear twenty-five years ago from the work of Painter and others; and only within the last decade have powerful techniques been developed for the further study of nuclei and of chromosomes, through isolated chromosomes, phase-contrast and electron microscopy, and more adequate chemical characterizations. With this extensive background and with more detailed treatments presented in this volume by others, a perspective on recent structural, chemical, and functional considerations may be helpful. We may start with the nucleus, keeping in mind Darlington's aphorism that "the nucleus is the chromosomes."

1. *On the Constitution of the Cell Nucleus*

MORPHOLOGY AND PROPERTIES. The oldest and the newest descriptions of the nucleus are based on observations of living cells. Favorable material, especially plant cells, was used with vital stains in the early work a century ago; the recent introduction of phase-contrast microscopy [384] increased the ability to discern structures in living material, broadening the base and improving the observations. From a long period of intermediate remoteness we inherit much information from studies of fixed, stained material [252]. Recent technical developments, as in microdissection, cinemicrography, polarization, and other forms of microscopy, have also contributed. For example, the presence of intact chromosomes in chemically hyalinized nuclei was indicated by D'Angelo's microinjections of suspended carbon [96]. A new era was opened with time-lapse moving pictures providing simultaneous records of structure and of function; this technique, so beautifully applied by Warren H. Lewis, has yielded many new facts, as from studies of the activities of cells in tissue culture [150, 159, 194, 221, 228, 288] that include descriptions of mitosis [195, 229, 236, 250], with details of chromosomal travel during anaphase permitting formal descriptive analysis [202] to complement earlier mathematical models [296]. When combined with recent developments in television, to permit high contrast imaging with much lower intensity of illumination, even greater accomplishments are in prospect. As a final example, the beautiful studies of birefringence by Inoué [198] have helped to remove all doubt about the reality of spindle fibers. Other promising techniques are developing, including abilities virtually to microdissect viruses [17, 168]. The best electron microscopy with thin sections has so far revealed the nucleus only as a relatively unstructured area [289, 314], but recent studies [18] of fixation by osmium tetroxide, so extensively used in such work, emphasize the need for more appropriate fixation of the nucleus. Such recent developments [197] thus hold great promise for increased understanding of the nucleus and chromosomes as vital elements in cell functions.

The nucleus [123, 194] is denser than the basic cytoplasm, the nucleolus being the densest part. Its viscosity is characteristically nonclassical and variable, reflecting sol-gel changes. The pH is generally thought (after Chambers) to be 7.6–7.8, with poor buffering power. The nuclear membrane generally carries a positive charge, unlike the cell membrane, and the chromosomes and nucleoli behave as though negatively charged. The isoelectric point of salivary gland nuclei of *Drosophila* has been determined by staining and by electrophoresis to be about 3.5. The isoelectric point of the nuclear membrane has been reported at about 11, indicating a role for basic protein. The membrane of nuclei isolated from frog oocytes was found by Holtfreter [185] to be characteristically different from the cell membrane (1) in its failure to repair mechanical tears, (2) in its toughness and thickness, (3) in its chemical reactions, and (4) in its chemical composition. Permeability to rather large molecules (molecular weight over 15,000) has also been seen [12, 185]. Microprobing within the nucleus may encounter no great resistance, but local gelations and dense aggregations may occur, much as seen in fixed material. Such changes may also be observed reversibly in cultured cells exposed briefly to vapors of formaldehyde, and clear living nuclei may often show heterogeneous structures on mechanical disturbance, asphyxia, or vital staining; such changes are generally reversible within limits. Just as in the striking condensation by electrolytes of chromosomes teased in nonelectrolytes [307], a dynamic role of electrolytes may well obtain for the nucleus [385] and perhaps condition many of its general properties.

When mitosis was discovered seventy-five years ago, the nucleus was seen to include a stainable filamentary substance as well as nucleoli, nuclear sap, and a nuclear membrane [252]. Morphological studies from the intervening years pertain to the form, size, and position of nuclei. The interphase nucleus may reflect the shape of the cell, being spherical in isodiametric cells, ellipsoidal in elongated cells, and flattened in squamous cells. Nuclei are of widely ranging size, but in a given cell type the nuclear size may be in rather constant relation to the amount of cytoplasm. Details of nuclear form and size have long been exploited, as in hematology; acute observations of this sort were well illustrated by Dr. C. H. Bunting in 1911 [64], when differential counts of leukocytes of healthy medical students were seen to show more lymphocytes and fewer neutrophiles than the accepted textbook picture. The position of the nucleus in the cell is also variable, but it is generally near the geometric center of embryonic cells and displaced with differentiation (as to a basal position in glandular cells). Nuclei may, of course, be lobated, multiple, or distorted, as, for example, in specialized cells (leukocytes, cells of spinning glands of insects) or in desquamated human epithelial cells [373].

Study of *living* animal cells usually indicates a nuclear membrane and a nucleus homogeneous save for characteristically refractile nucleoli and chromocenters that represent heterochromatic, persisting segments of the chromosomes. Some interphase nuclei have a granular aspect, while others show granules united by a reticulum (cf. Serra's classification of dispersed and thread-type nuclei [328]. Plant nuclei [113] show similar degrees of reticulation in both vital and fixed conditions. The characteristic emptiness (anisotropy) of nuclei noted by von Ehrenberg in 1848 has persisted under newer techniques, including the ultramicroscope and recent work showing diffuse ultraviolet absorption by interphase nuclei [307]. Optical homogeneity does not, of course, necessarily indicate structural homogeneity. The chromosomes persist in some form, and the consensus now seems to be that interphase chromonemata represent the chromosomes in a highly hydrated or dispersed colloidal state. The negative birefringence of sperm heads gives indirect evidence of interphase nuclear order, but most interphase nuclei are not birefringent. This anisotropy could reflect concentration of oriented nucleic acid, with ester chains parallel to the fiber axis. Such orientations have been reported [276] for stretched interphase plant nuclei. Nonheterochromatic parts of chromosomes are occasionally visible in living interphase chick and mammalian nuclei as very fine chromonemata [145, 146]. Interphase chromosomes have also been well demonstrated for some insects, especially the heteropyknotic sex chromosomes [157]; the permanence of the chromosomes of some protista is striking [81, 82]; and recent evidence shows interphase chromosomes in living avian material [194]. Chromosomal permanence in higher forms is also indicated by the correspondence of the chromocenter with heterochromatic regions [123].

In studies of the state of the chromosomes in the interphase nucleus [307] the cited diffuse ultraviolet absorption and a diffuse Feulgen staining were seen either *in vivo* or after fixation with formalin or osmic acid. Chromosomal images in ultraviolet micrographs of living grasshopper spermatocytes and onion epidermis were suggested only when visible images were seen with ordinary light. An extended state—with uniform distribution of deoxyribose nucleic acid (DNA)—and a condensed state—with localization of DNA in typical chromosomal structures—were therefore recognized. Teasing nuclei in 10 per cent sucrose gave the "living" appearance, while in physiological salt solutions the nuclei had the "fixed" appearance; chromatin is evidently extended in nonelectrolytes and condensed in electrolytes, and the interconversions may be reversible. The chromosomes teased from nuclei in glucose or glycerol stained homogeneously unless salts were added, when they condensed and stained with methylgreen differentially. Salt-isolated chromosomes when resuspended in sucrose increased four- to fivefold in volume, and removal of DNA and of histone destroyed the capacity to extend and to condense reversibly. These reversible condensations, as seen earlier for isolated nuclei [385], are also effected by salt concentration, paralleling the behavior of isolated nucleohistone [258]. According to Pollister [282], the distribution of protein is also diffuse in the extended state. An indication of a central role of DNA in the reversible extension may be seen in the failure of lampbrush chromosomes, poor in DNA, to behave in this fashion.

When nuclei are *fixed* (and White [370] refers to the interphase nucleus as "unfixable"!), fine, twisted basic filaments (chromonemata) in a reticulum with other finer filaments (linin) may be seen along with dense flakes of acidophilic chromatin (chromocenters or karyosomes, false or chromatin nucleoli). This well-known appearance of some fixed nuclei was expressively described by Dr. C. H. Bunting [65] a few years ago for the polymorphonuclear leukocyte as "a *basketwork* of deeply staining chromatin strands." The chromatin may also be either dustlike, heavily granular, or applied to the nuclear membrane as a continuous layer [55], the variability being emphasized according to the fixative. Osmic acid, for example, is associated with nuclear homogeneity, and mercuric salts with coarse chromatin and detailed reticulum. The similarity of fixed nuclei to coagulated colloids and the variation in their appearance with fixation led to considerable skepticism concerning the reality of the interphase aggregates of fixed chromatin. More recently a plastic reticular network of strands has been pictured [151], this being held invisible in the living state and apparent only on dehydration or fixation; and a fine irregular reticulum with 30–80 A particles has been described as a primary nuclear structure [56]. Such views represent efforts to understand the interphase nucleus; Barigozzi, however, in a recent review [24] concluded that no general model is yet available.

Two special types of nuclei may be mentioned for perspective: those of bacteria and of oocytes. Divergent views concerning bacterial nuclei have ranged from doubts of their very existence to the view that bacteria are simply naked nuclei [53]. Bacterial nuclei have been described for a number of micro-organisms, with examples interpreted as illustrating all stages of mitosis [111]. Some workers have disagreed with these interpretations, attributing them to a mistaken multinuclear nature in actually multicellular bacterial forms [37–39] or to artifacts produced by organic solvents [166]. Nonetheless, mitosis in micro-organisms is reported for a number of forms [42, 106, 107, 111, 112, 135, 310, 354, 355]. DeLamater [108, 109] recently summarized his formulation of the structure and division mechanism of the bacterial nucleus. The chromosomal threads studied are admittedly near the limit of resolution, but all stages of this mitotic schema have been well illustrated by micrographs. The action of some drugs was found helpful; para-amino salicylic acid, for example, brought out an apparent centriolar inhibition, and several antibiotics (terramycin, aureomycin, and streptomycin) produced apparent metaphase inhibition and polyploidy. Concerning the alleged confusion with bacterial walls, DeLamater reported a new double stain demonstrating both septa (red) and nuclei (blue) in a given cell, apparently justifying his position. A companion paper [253] reported effects of colchicine on the mitotic apparatus of *Salmonella typhosa,* with responses suggesting a true mitotic apparatus. Such work would be forwarded by isolations of nuclei from bacteria; so far, only one unconfirmed report has claimed isolation of chromosomes from bacteria [238]. Phase-contrast microscopy has evidently revealed only clear nuclei in living bacterial cells, so far. Using clear Feulgen-stained plates, some workers have checked chromosome numbers for micro-organisms [136]; a rare separation of a chromosome into three instead of two chromatids during mitosis has been reported for two strains of yeasts [134]; and fungal nuclei have been studied [249, 267]. One recent study [79] emphasizes the need for making combined studies with stains for both nucleus and cell wall, to avoid confusion from configurations simulating mitosis.

The obvious application of electron microscopy of thin sections of bacterial nuclei has just been undertaken [175] and high resolution micrographs of E. coli during the growth phase [36] have shown axial "vacuoles" with no evident membrane but with scattered, folded "threads." In the resting phase these vacuoles are represented by winding ducts, each containing a single thread 250 A in diameter. The "vacuoles" correspond to the "nuclei" of stained preparations. A shift in the threads that parallels shift in DNA-staining after infection with T4 bacteriophage has suggested that they contain DNA. Thin sections and a modified Feulgen technique with silver depositions [53] have confirmed earlier indications of bacterial nuclei containing structures resembling chromosomes and nucleoli. Other electron microscopic studies include observation of the electron transparency of yeasts following ultraviolet photolysis [25] and studies of E. coli [164]. Important new findings are to be expected in this area.

Other nuclei and chromosomes favorable for study are found in amphibian oocytes [155]. These develop in the follicular epithelium, having initially the chromomeric appearance of early meiotic prophase. As prophase proceeds and the nuclei attain a diameter of 40 micra, lateral projections arise conferring a rough (lampbrush) appearance to the chromosomes. Multiple nucleoli are seen here against the nuclear membrane when the lateral loops appear on the chromosomes; hundreds of nucleoli are so situated later, while others form radial lines and a dense coat around the chromatin mass. Extrusion of such nucleoli into the cytoplasm, with its important functional implications, has been described by Painter and Taylor [270] and by Duryee [132], and the lampbrush chromosomes have been studied to good advantage [154, 216, 306].

Continuing our anatomical considerations, the *nuclear membrane* [75, 123, 151, 194] was studied with polarized light by K. von Erlach in 1847 [139] and described as comprising concentric annuli and perpendicular birefringent fibers. Baud [26, 27] accentuated the birefringence of this membrane by impregnating fixed liver cells and histiocytes of rat, dog, and cat with antimony sulfide. This revealed in the "achromatic" nuclear membrane a birefringence negative with respect to the radius that was unaltered by removal of lipides and hence indicative of protein sheets or micelles parallel to the surface. The perinuclear layer was also noted to have a positive birefringence (attributed to lipides) that becomes negative (protein) after lipide extraction and was hence characteristic of lipoprotein normal to the protein-layered surface. Nuclei swollen in "isotonic" media [67] displayed a retardation negative with respect to the radius, as found by Baud, that was proportional to the diameter and hence to tension. This birefringence disappeared in a medium with refractive index of 1.5, indicating a protein form birefringence. Randall [295] described electron microscopic studies of oocyte nuclei of two amphibia, distended and ruptured with 0.8 per cent NaCl, that showed for *Xenopus laevis* an external porous layer and an internal, continuous membrane. The pores were about 400 A in diameter spaced 1000 A between centers, and the porous layer was reported to contain some lipide. Similar results were obtained for *Triturus* and *Xenopus l.* [69], with 300 A pores spaced 800 A between centers, in an outer membrane 300 A thick (fixed), supported by a continuous membrane 150 A thick. These findings were confirmed on other material (oocyte and neuron [56]), 70 A pores being seen at regular intervals. Some microdissection and electron microscopic studies of nuclear membranes [47, 372] have indicated that for several insects oocyte membranes are simply porous, with regular annuli 100 A in diameter; no continuous membrane was seen after fixation in osmic acid. The membrane of the 75 micron nucleus of the protozoan parasite *Gregarina acridiorum* was found sensitive to treatment, but fibrillar and porous structures were seen. Nuclear membranes of salivary gland cells of *Chironomus* were found simply porous, while fibrillar structures were seen in two strains of amoeba.

All of these appearances were dependent on stretching and on medium; the holes and fibrils were believed to be true structures, but the annuli were considered suspect. For the nuclear membrane of *Amoeba proteus,* a protein layer and a porous layer with 1200 A holes have been indicated [20]; juxtapositions were not clear from these studies by fragmentation. Contrary to the orientations reported above, later sections [169] of this same material have shown an *outer* continuous layer 1000 A thick and an inner 2000 A layer bearing 800 A pores on 1200 A centers. More recently, Gall [156] isolated nuclei from oocytes of *Triturus* by microdissection in 0.1 M NaCl, broke and fixed them, and dried them for electron microscopy either in air or by the critical freezing-point technique. A fixed, wet preparation by phase-contrast disclosed circular areas about a micron in diameter with internal granulation. In electron micrographs the granulation within these circular areas comprised clusters of smaller granules (1200 A), which at higher magnification appeared as regular annuli comprising 8–10 smaller masses, each about 400 A in diameter. These annuli were believed to be artifacts after air drying; after critical point drying only masses of spheres were found. Although confirming the earlier results [69], the annuli were believed to represent discrete specialized areas rather than breakdown products of a uniformly porous layer. In addition to reports of nuclear membranes with porous and continuous layers in both relative orientations, work with salivary glands and the midgut of larvae of *Chironomus* confirms the existence of porous nuclear membranes with *no* continuous layer [19]. The 500 A pores here, spaced on 800–900 A centers, are clear in cross sections and tangential sections. The membranes were about 300 A thick, with two layers of 70–80 A each; the rims of the pores were marked in the midgut by heavily absorbing material. Rhodin [301] in perhaps the first such observation for mammalian cells, has shown regular ringlike formations about 900 A in diameter in tangential sections of the nucleus of cells from the kidney of the mouse; these may correspond to the porous structures observed by others, including examples for sea urchin oocytes [1]. Porelike structures, 200 A to 300 A in diameter traversing the nuclear membrane of pancreatic cells in the mouse, were recently reported [365], but 800 A "pores" of the nuclear membrane of chicken embryo cells are noted as special structures rather than as simple openings [209]. A recent extensive paper has emphasized the generality of pores in the nuclear membranes, with a perinuclear space being indicated by some continuity of the outer nuclear membrane and endoplasmic reticulum [366]; this suggests two channels of contact, direct and indirect, between nucleus and cytoplasm. Thread-like structures continuous from nucleus to cytoplasm have also been noted in early frog oocytes [283, 284]. Some workers have expressed general reservations about the degree of permeability of the nuclear membrane, especially because of uncertainties concerning the effects of isolation [180], but from all of the foregoing one sees adequate if varied structural basis for a physiologically active nuclear membrane.

It is unfortunately not as yet possible to discuss the *nuclear sap* in much detail. This component can be demonstrated in some instances by perforating the nuclear membrane, a fluid flowing forth. Two phases have been distinguished here [68] by pH tests, one a rigid structural colloid, the other a dispersed fluid colloid with primary osmotic reactivity. A negative colloid is shown by hydration experiments, minima occurring with potassium. Viscosity as discerned from rate of fall of starfish nucleoli has shown a Q_{10} of 2.7 for the range 0–35 degrees C, the viscosity decreasing with successive repetitions [367]. The contents of the germinal vesicle thus behave as a thin thixotropic gel. Not much of this "nuclear sap" can exist in some nuclei: D'Angelo [96] found that the chromosomes can crowd much of the nuclear volume. Indeed, it may be unfortunate to consider this "sap" as a component separate from the chromosomes.

Although perhaps the first intranuclear structure to be described, the *nucleolus* now holds special interest because of recent developments concerning its structure, composition and function [2, 83, 123, 148, 151, 194, 230, 232, 298, 299, 322, 334, 341, 360]. Recent morphological studies of oocytes include extensive work with arthropods [144], triton and frog [165, 270], and studies of newt oocyte nuclei [155] that contain many extrachromosomal nucleoli, possibly heterogeneous and not wholly homologous with the nucleoli of somatic cells. These remain under investigation, especially as to origin and significance. Recent accounts of the bodily movement of nucleoli through the nuclear membrane include, in addition to those cited, examples for amphiuma and *Squalus* [124], for teleosts [77] and for amphibia [132, 270, 374], and such reports based on sections are paralleled in other material but suspected as artifacts of displacement by the microtome blade [155]. Duryee [132], however, reported having watched the extrusion of nucleoli through the nuclear membrane.

In 1948, DeRobertis, *et al.* [122] described the nucleolus as the Feulgen-negative, ribonuclease-positive, densest nuclear component—visible in the living cell, negatively charged as judged by migration, absorbing ultraviolet light of wavelengths 2,600 A and 2,800 A, with a probable role in protein synthesis (Caspersson); in mitosis it was generally known to disappear at prophase with the nuclear membrane and to reappear at telophase; and its composition reportedly varied with rearrangements or with sex differences in *Drosophila melanogaster.* Other descriptions [151] are less helpful: "An accumulation of karyolymph in a coacervate droplet rich in proteins." In 1954 DeRobertis, *et al.* [123] described the recent work on the nucleolonema which brings into question the relation of nucleoli to special chromosomal regions. This Feulgen-negative filamentous structure is reported to be permanent, distributing along the chromosomes at prophase and following the chromosomes through division to reassemble after anaphase into new interphasic nucleolar form [142]. Some parallel behavior has recently been reported for nucleolar zinc [153]. The relation of nucleoli to cytoplasmic ribonucleotides or ribonucleoprotein and to protein synthesis is further suggested by more evidence of nucleolar extrusion in oocytes, by contrasts among lymphocytes of various sizes, and between liver cells on various diets, including controlled protein intake [123]. The ribonucleoprotein nature of the nucleolus has been confirmed by work with isolated starfish nucleoli [360], in contrast to other recent reports [232].

Recent morphological studies of the nucleolus [140, 141, 332] suggest that it has the structure of a network, and electron microscopic work [30, 31, 45], has revealed filamentous and granular structures. These have also been found recently in living normal and malignant human cells in tissue culture [119]. Nucleolar-organizing chromosomes of Spirogyra and nucleoli with coiled structures and elongated chromosomal tracks were illustrated by Godward [161]. Bernhard, *et al.* [31] reported tangled filamentous elements 90–180 millimicrons wide in the nucleoli of a variety of mammalian cells (rat liver and other tissues, mouse mammary carcinoma, human kidney); these were Feulgen-negative and therefore considered to contain the ribonucleoprotein of the nucleolus. Our observations in living human material after mild treatment with hypotonic salt solution emphasized the reality and interest of the nucleolonema, seen previously only by special techniques (silver impregnation or electron microscopy) or in special materials. The field is not without current uncertainty, as in the recent report of Feulgen-*positive* nucleolar filaments [223, 224]. Studies of the nucleoli in various functional states are being undertaken [186]. Feulgen-positive filaments associated with dispersed human nucleoli have been seen in our laboratory, but a crucial localizing experiment, as with stained sections, is needed.

In passing, it is noteworthy that sexing of tissues may be possible from details of nucleolar morphology. Thus a so-called sex-chromatin mass associated with the nucleolus has been claimed unique for the female [15, 16, 262, 263]. This sexing has been applied to important medical problems as of hermaphroditism and the etiology of teratomata [196]. However, this chromatin was found by another careful study *not* to be unique for the female [85]. Another recent observation claims accurate sexing from a solitary 1.5 micron "drumstick" nodule on the nuclei of human neutrophil leukocytes [102].

CHEMICAL CONSTITUTION OF THE NUCLEUS. The general composition of nuclei was indicated in 1875 by Miescher [251], who reported that the heads of salmon sperm contained 61 per cent nucleic acids, 20 per cent protamine, 2 per cent lipide, 0.2 per cent calcium, and a 17 per cent residue, probably protein. More recent data for isolated somatic nuclei and isolated chromosomes, chiefly bovine, have indicated about 35 per cent DNA and 2 per cent RNA, 30 per cent basic protein and 10 per cent to 50 per cent residual protein, about 15 per cent lipide, and a few per cent ash. Thus nucleic acids, proteins, and lipides figure importantly in chromatin, and the newer data disclose RNA and a special tryptophan-containing residual protein. Such data from isolates in aqueous media suffer uncertainties, as from protein losses during isolation [7, 61, 127, 212, 286], but studies of methods of nuclear isolation continue [93, 131]. Thus it appears that damage to mitochondria during isolation may condition enzymatic release of protein from firmly bound nucleoprotein [130]. Contamination of some nuclear fractions with thymocytes was recently suspected from indirect studies of effects of ionic environment [60] but minimized on other direct evidence [6] which did indicate a small cytoplasmic contamination of such preparations. A promising, clean separation of discrete fractions from tissue homogenates has been recently reported by centrifugation with constant density gradient [13] and a heartening similarity in incorporation of labeled precursors of nucleic acids was recently reported [101] for nuclei isolated either by the citric acid or Behrens techniques. Such work serves to increase confidence in the results of chemical characterization of isolated nuclei.

The structure and the chemistry of DNA, especially, have recently been forwarded [4, 90–92, 105, 273]. Analyses for the nucleic acid contents of individual nuclei and amino acid analyses for some of the proteins have been reported, and although largely neglected, the ranges for lipide compositions are now clear. A reasonably consistent if fragmentary picture is thus developing [280, 281], and questions of method, with emphasis on combined direct and indirect approaches to localizations, are being clarified [285]. Recent considerations [231, 268] of the effects of scattering of stray light (the Schwartzschild-Villiger effect) have led to new apparatus [103, 264, 265], and comparisons of biochemical and microspectrophotometric results have been reassuring [225]. Questions of the effects of media on analysis as of isolated nuclei are being developed [14, 321] but the interpretation of chemical and enzymatic analyses of isolates still requires caution. Nuclei are unquestionably altered by unnatural environment (cf. the basic experiment of Comandon and de Fonbrune [89]). Mazia [243] in a helpful review emphasized the dual chemical organization of chromosomes into fractions containing DNA together with basic proteins, and RNA and residual proteins; intermolecular associations of the fractions in the nucleus remain to be worked out.

The proteins of the nucleus, early recognized to include protamine (Miescher) and histone (Kossel), are now being fractionated and characterized by several workers using chromatographic, ultracentrifugal, and other kinetic techniques, with exciting, provocative findings. Although some such results emphasize the diversity of composition of such proteins, a constant ratio of arginine to DNA has recently been reported for isolated nuclei of several fish, one bird, and one mammal [358]. Lipoprotein has been included in the fractionations, as of mammalian sperm heads [72, 94, 351, 363], phosphoproteins are of interest [205], isolated globulins and protein fractionations have been reported for nuclei [137, 212, 213], and both basic and acid proteins have been separated, with indications of heterogeneity in some basic proteins (thymus histone [163, 333]). The conjugated nucleoproteins have also been receiving much attention [87, 104, 282, 347]. Uncertainties of techniques have obscured simple patterns, and relations as of RNA synthesis and protein remain unclear, but real advances are being made by cautious chemical comparisons. For example, adequate confirmation of the theory of constant DNA contents has developed. Linkage as of the DNA-histone complex in salt isolates may not obtain *in vivo,* but developing knowledge of such complexes [258, 261] promises functional roles as for residual protein. A few studies of the proteins of the karyolymph [61, 151, 339] have indicated cyclic amino acids, with complex composition, and absence of nucleic acids (for preparations freed centrifugally of chromosomes and nucleoli); such work suggests the ripeness of this field for further chemical analysis.

The special nuclear proteins with enzymatic activity have been under intensive study for years, but Dounce in a review of results for isolated cell nuclei emphasized the confusions and technical difficulties of this field, factors such as extraction, adsorption, and nuclear permeability to protein complicating the determinations [129, 184, 291]. Glycolytic and protein-synthetic systems and alkaline phosphatase have been indicated for several kinds of nuclei, with oxidative systems deficient [125–127, 219, 338]. An isolation medium can affect such results markedly, and uncertainties of contamination of nuclei with whole cells or cytoplasmic constituents mar such results [182]. Nuclear concentrations of catalase and arginase were indicated [337], and reports of many other special enzymes have appeared. Histochemical localizations of phosphatases were recently brought in question by the evidence of diffusion of calcium phosphate before its precipitation [128, 266]. The simple expedient of staining before removal of paraffin from sections [162] has helped clarify the error of some nuclear stainings for phosphatase. With proper fixation and controls, the Gomori technique can show some evidently strong nuclear localizations of this enzyme that undoubtedly correspond to the living nucleus [78], possibly in a role relating to mitosis. The analytical approach to nuclear enzymes developed hazards as of contamination but recently indicated a clear localization in isolated liver nuclei of an enzyme for synthesis of diphosphopyridine nucleotide (DPN) [181]. This is especially interesting because the enzyme system linked with DPN is associated with liver cytoplasm, and nuclear supply of a co-enzyme is suggested. Other workers have also been identifying a number of enzymes in isolated nuclei [331]. It appears clear that despite the technical uncertainties we stand ready for important gains in understanding of the biochemistry of the nucleus.

Lipides, as mentioned, were reported in the earliest analysis of sperm

[252], and the more recent data verify the substantial proportions and suggest protein bindings, with associated RNA as in nucleoli [125, 194, 359]. Phospholipides have been indicated [2], and staining of intranuclear lipides has suggested structural and metabolic activities [59]. The fragmentary data for these substantial nuclear components represent a great challenge.

Inorganic and other constituents of the nucleus have not been neglected through the years. The ash contents were analyzed at first chiefly by microincineration. Despite uncertainties of migration and identification, such analyses have shown many cations and minerals, with provocative reports as of nucleoprotein-bound copper and of nuclear concentration of zinc [187]. More recent nuclear localizations of radioactive cobalt, copper, and other minerals have brought new promise to this field of analysis [292, 309]. Among the varia reported, the nuclear concentration of materials related to B vitamins is provocative [125], and there appears some evidence that, despite many negative reports, a role for carbohydrate may be seen in the extrachromosomal division apparatus of certain cells (Acetabularia [340]).

The foregoing suggests some general perspective of the chemical constitution of the nucleus: this area is technically difficult but unfolding, and there are many indications of significant metabolic activities hinging upon nuclear constituents.

2. *On the Constitution of the Chromosomes*

STRUCTURAL AND GENERAL ASPECTS. Chromosomal form and function have been the subject of many excellent reviews, and the characteristic complements for particular cell types have been detailed by number, dimensions, form, behavior, and internal structure [74, 86, 100, 123, 151, 158, 194, 207, 208, 234, 241, 242, 322, 370, 371]. Although some zoological groups show peculiar karyotypes, as in the telocentric chromosomes of locusts and the metacentric chromosomes of some amphibia, size is relatively constant in a given state and may range from 0.2 to 50 micra for length and 0.2 to 2 micra for width. The human karyotype had been competently investigated even in somatic cells [210], but it was rather poorly known, surprisingly, until Hsu [189] capitalized on the excellent displays effected by hypotonic salt solutions to derive detailed idiograms showing most of our chromosomes as J's, the largest pair or two and the smallest unmatched chromosome (presumably Y) being V's. Such detailed studies [192] of chromosomal complements are proving useful in work with cancer cells [171, 190, 191, 226, 227, 353], a field of particular interest because of the evident nuclear role in the heritable trait of malignancy. The *numbers* of chromosomes, long studied systematically, have been under scrutiny in recent years [193], especially following reports of wide variations, including modes in subdiploid ranges [350]. Sachs [315] found that scorings for squashes of undamaged endometrial cells did not indicate such variations, however, and other workers [362] have reported such variations only in squash preparations of tissues other than sheets or dispersions of cells. Squashing is evidently capable of rupturing groups of chromosomes.

The *fine structure* of chromosomes, as generally described, includes a membrane, a matrix, and granular chromomeres, with probably common chromonemata; the helical or particulate nature of chromomeres has recently been under scrutiny [155, 176, 216, 302, 303, 306, 312]. Multiple chromonemata are generally believed present. Indications of this have been seen in amphibian and in avian material [177, 379]. Helical coiling of the chromonemata has been clear since the work of Baranetsky with Tradescantia in 1880 [23], and is seen in many forms, including mammals [235]. Detailed considerations of this coiling, supplementing earlier reviews [343], have recently appeared [81, 237]. Questions of the reality of the minor helix [312], superimposed on the clear major helix, leave uncertain the generality of the double-helical structure. Although further work with special forms is needed, in some forms the minor helix seems perfectly clear [88]. The favorable material employed by Cleveland [81] has added much detailed knowledge of coilings and their behavior through the division cycle, and resolution of the disparate reports for coiled structure in lampbrush chromosomes now seems at hand. In a recent review, Ris [305] found by electron microscopy of several favorable materials that bundles of 500 A to 600 A microfibrils underlie the structure of prophase chromosomes, with smaller (350 A) fibrils suggested for interphase as a basic unit of structure. The desoxyribonucleoprotein recently isolated from sea urchin sperm [32] perhaps correlates with this pattern, and Mazia [245] has discussed the particulate organization of these macromolecules, perhaps ionically bound.

Isolated chromosomes prepared from interphase nuclei just over a decade ago by Claude and Potter [80] are especially important because of their convenient availability in bulk. The chromosomal nature of these isolates was suggested by the pioneering studies, and developed by Mirsky and collaborators in some detail, including chemical characterizations. The identification of these important isolates with the chromosomes was questioned [70, 217, 218], however, on the belief that the isolates were merely random bits of drawn nuclei or of nuclear reticulum; such objections have been viewed seriously by some leading workers [194, 317]. Careful studies [114–116, 275, 278] have nevertheless indicated that these isolates do indeed represent the chromosomes. Using intermitotic nuclei as a source of isolated chromosome threads from cultured muscle and epithelium of Drosophila, Pfeiffer [275] concluded from studies of staining and of birefringence that these threads were true intermitotic chromosomes. Distributions of the lengths of the isolated chromosomes were established by Polli [278] for a wide variety of biological materials, for comparisons with the lengths seen *in situ* by classical techniques; the good correspondences for the varied complements studied made clear the identity of the isolates with the chromosomes. Our data [114–116] included similar and detailed studies of the form and size of isolates from chicken red blood cells, and in addition to finding isolated chromosomes in shapes and sizes anticipated from the data for the metaphase chromosomes, two crucial objections were found invalid. Thus detailed studies indicated the absence of any important effect of the speed of the Waring Blendor on the lengths of the threads, and provided an alternative interpretation for drawn nuclei. These were found to be virtually absent from fresh wet mounts for phase-contrast microscopy and present in high and constant proportion in dried mounts for electron microscopy. The proportion of drawn nuclei was independent of the time of treatment by either of two radically different means, and the distorted nuclear appearance was reproduced with whole cells by osmotic abuse and drying, as in the preparations for electron microscopy. The association of such isolates with the chromosomes of the interphase nucleus thus appeared clear, as indicated from such grounds by Ris and Mirsky [308]. Supporting evidence is also available from studies of Waring Blendor preparations of isolated chloroplasts from tobacco leaves, whole nuclei and Feulgen-positive strands being seen as contaminants, the actual issuance of some of these strands from the nuclei being reported as evidence of their clear nuclear origin and derivation from vegetative chromonemata [368]. Obviously drawn nuclei were also seen by these workers, but such artifacts should be readily recognizable. Extrusion from interphase nuclei of filaments with the appearance of isolated chromosomes has also been attained by pressing (Mottram and Denues, unpublished). Some interesting detailed results have also come from cytochemical and electron microscopic studies in Japan [378, 381, 382]. These included isolates from erythrocytes of *Clemmys japonica* [381], showing beautifully structured objects interpreted to support earlier conclusions of the fine structure of isolated chromosomes. A serious problem from contamination with micro-organisms mars such findings, however (as also appears likely in another instance [118, 279]); the appearance of such contaminants has been documented [120, 188] and now appears generally appreciated [383]. Alfert [3] in a recent review referred delightfully to the danger of isolated chromosomes looking "too much like chromosomes"! Some bacteria with such helical internal structures have been identified by Xalabarder [375–377] as Mycobacteria, but full identifications of such confusing organisms remain to be worked out.

With the evidence indicating clear association of isolated chromosomes with interphase chromosomes, the results of the many studies with these isolates are assured of great interest. As examples, knowledge of chemical composition, including lipides, was derived a decade ago with isolated chromosomes [80, 240], and the later chemical characterizations of isolated chromosomes have even suggested the functional role of the residual protein [259, 260]. There is evidently much fine structure in isolated chromosomes to be developed [117]; studies with isolated lampbrush chromosomes are also of great interest [155, 216, 295].

Another approach is with *giant chromosomes,* the lengths of which extend to tenths of a millimeter (several hundred times the length of usual somatic chromosomes). Discovered by Balbiani in 1881 [21] in salivary glands of larvae of the fly *Chironomus,* the amazing, fine structure of such chromosomes was developed in the early thirties by Heitz and Bauer [174] (working with *Bibio*), and by Painter [269] (working with *Drosophila*). The characteristic Feulgen positive bands of such chromosomes, strongly absorbing for ultraviolet light and for electrons, have been exploited for genetic mapping and for detailed morphological studies [272, 325]; various special techniques have been applied to them, including microdissection [62, 96, 97] and electron microscopy [44, 272, 325, 380]. Knowledge of their physical properties has thus developed and has indicated polyteny. A helpful positioning review by Alfert [3] has noted the various interpretations of the bands of these chromosomes as well as the absence of longitudinal striations in replicas and sections of unstretched chromosomes studied by electron microscopy; submicroscopic fibrous structure is believed clear, however, from evidence derived with stretched chromosomes. Studies of birefringence and of dye binding [9, 274] have also contributed, and Alfert considers the polytene theory best supported by available evidence [cf. 28, 215]. Films showing infrared or ultraviolet dichroism [311], negative birefringence, and marked anisotropic (lateral) swelling in water, similar to oriented fibers of nucleic acid, have been prepared recently by stretching moist nucleoprotein from herring sperm on a rubber base [8]. Similar lateral swelling, exceeding longitudinal swelling by two and one-half fold, with banded regions showing greater anisotropy, was also seen in mounted salivary glands [9]. Orderly, folded longitudinal chain molecules are indicated by such behavior, as required by the great extensibilities found by D'Angelo's microdissection work. The ultraviolet dichroism reported [311] and the recent experiments with fresh salivary gland chromosomes, showing lateral swelling and dichroism [10, 11] also suggest longitudinal structural elements. Other studies with newer techniques, such as X-ray absorption [138], have appeared, and a provocative variability of the banding patterns has recently been emphasized [326, 327].

CHEMICAL ASPECTS. Developments in the chemistry of the chromosomes and nucleoli have recently included the indications of constancy of chemical contents of DNA, as for nuclei, with doubling prior to division and geometric increase with ploidy. The isolated chromosomes have been found to comprise DNA, histone, residual protein, and RNA, with relative constancy in the first two but variations in the last two correlating with the physiological activity of the cell. Important studies of the interphase state of the chromosomes [307] and fractionations, similar to those mentioned for the nucleus [3], have also been reported [cf. 147, 254, 304, 329]. Although cited losses of material or contaminations from cytoplasm may limit the analytical use of isolated chromosomes, their bulk is clearly nucleoprotein, and the presence of lipides, carbohydrates, calcium, and magnesium, as commonly reported for nuclei, appears clear. Ris [304] notes that the protein includes simple histones and protamines, nonhistone protein containing tryptophan, residual nonhistone protein insoluble in molar sodium chloride, and fractions cited before. An active, large cytoplasm has been found associated with a high content of nonhistone protein [307].

The nucleoli are especially interesting from a chemical standpoint—proteins, alkaline and acid phosphatases, RNA, and high 2,600 A absorption being indicated, along with Feulgen-positive granules shown by Painter and Taylor [68, 270]. There has been some confusion here concerning the presence of DNA in isolated nucleoli [232]. Vincent [360] noted a largely phosphoprotein composition for isolated starfish nucleoli, with significant RNA and small amounts of lipide and soluble nucleotide but no histone. Acid phosphatase was the only enzyme detected, possibly because of contamination. A primary RNA and protein composition for the nucleolus was reported by Schrader [322] and the predominant importance of basic protein was cited by Caspersson [73]. A negative result for acid phosphatase was also reported [294]. The positive results reported by other workers were obtained only for chromatin associated with the nucleolus. Feulgen-negative nucleolonemata were seen by Bernhard, *et al.* [30, 31], while Lettré and Siebs [223, 224] have reported some Feulgen-positive filaments within the nucleolus, presumably chromosomal in nature. Both filaments and 150 A uniform granules supposedly rich in RNA have been pictured in striking nucleoli of larvae of Ambystoma by Porter [290], the granules being presumably similar to Palade's granular component of the cytoplasm, associated with basophilia. Special stainings have brought reports of phospholipides [2] and of zinc [152, 153] in the nucleolus and nucleolar chromosomal zones, and the presence of autolytic enzymes and alkaline phosphatase in whole and residual chromosomes has been noted [256]. A recent report cites enzymes (nucleoside phosphorylase and cozymase-forming enzyme) and substrates (guanine, 5-adenylicacid) that may support active metabolism in starfish nucleoli [22]. The chromosomal proteins have shown some differences in amino acids [95]. Such activities suggest that, as with the nucleus, a fragmentary but developing picture of the chemical constitution of chromosomes is now being gained [257].

3. On Nuclear and Chromosomal Functions

The commanding position of the nucleus was clear a century ago to Virchow, who appreciated that the nucleus was more concerned with the maintenance and multiplication of the cell than with its specific function. Although the transitory existence of cells after loss of nuclei was recognized, Virchow suggested that no part grows or multiplies without starting from nucleated elements. These concepts, generally supported by later work, have been formulated as a dual nuclear function [243, 247, 338] concerning either roles essential for the life of the cell itself or for the cell-line, as preserving a code of inherited characters. Recent studies with experimentally enucleated cells have emphasized the independence of some activities of the nucleus, but both immediate and longer-term functions for the nucleus are clear.

Roles in *heredity and reproduction* represent longer-term functions of the nucleus [300, 335]. These roles have a long history rooted in the details of fertilization and early recognition of the particulate nature of heredity and localization of hereditary particles in the chromatin. The exact correspondence between the distribution of chromosomes and of genomes [345] emphasized the common essential features of cell division and of heredity, namely purity and independent transmission of units (chromosomes and characters). An unusual emphasis on the reproductive role of the nucleus appears in certain uninucleate algae, as in *Acetabularia,* in which at maturity small nuclei form and become spores for propagation of the species [167]. Various theories of heredity bearing upon the nuclear role have developed [43, 66, 151, 344, 371]. The genetic function of the nucleus is usually referred to the "chromatin," and DNA has been extensively considered an attractive candidate for the genetic role because of its constant amount in diploid cells; proportionality to ploidy, specificity, variety; and true genelike transforming action. Another view, involving a hereditary role for RNA, has recently been championed [239]. The dependence of chromosomal integrity on the combination of DNA and residual protein is notable [259, 260]. A molecular model of the gene (a biological *concept*) as a fundamental molecular unit of chromosome function has appeared plausible under the scrutiny of Schroedinger [323], and the recent indication of equal masses in normal and mutant sections of salivary gland chromosomes [313] suggests a small-scale (molecular) rearrangement as the basis of mutation.

Mutual *interactions of nucleus and cytoplasm* in growth and in development characterize the shorter-term functions of the nucleus. Interdependence of the parts of the cell is clear from revitalization and even perfect larval development of refertilized, enucleated eggs [170]. Such interactions are also apparent from labyrinthine nuclei of some large cells [54, 371], and from studies with *Acetabularia* [167]. A suggestion of nucleo-cytoplasmic interchange in dividing cells was recently seen in the relatively greater density of the ground material immediately around the nucleus of growth-phase E. coli [36]. In addition to the central role of the nucleus in development, activities of the interphase nucleus include the striking nuclear migration, eversion of nucleoli, and lampbrush formation in oocytes [133]; nuclear rotation [288, 349]; and chemical nuclear differentiations that suggest direct interactions of cytoplasm and chromosomes [255]. Most recently, parallels in response of cytoplasmic and nuclear protein fractions of certain tissues of adult mice following feeding and fasting have been seen from uptakes and retentions of N^{15}-glycine [5]; these indicate a correlation of syntheses of protein in the cytoplasm and in the chromosomes, and a pattern of interaction between cytoplasm and chromosomes is suggested for nondividing cells. Nuclear coupling of oxidation and phosphorylation was suggested [48, 49, 204], and catalysts (possibly zinc [153]) presumably released by the chromosomes may govern cell division [346]. A replacement hypothesis which provides for nuclear supply of enzymes, particulates, or integrated units for cytoplasmic working mechanisms is an attractive possibility [243], correlated well with the gradient of morphogenetic capacity discovered in *Acetabularia* [167]. In rapidly growing malignant cells a concentration of filamentous mitochondria opposite chromosomes applied to the nuclear membrane suggests nucleo-cytoplasmic interplay [233]. Adequate permeability of the isolated nuclear membrane for materials such as proteins has been indicated [185], but definitive uptake studies are needed. Nucleocytoplasmic relations, as reviewed recently [143, 324, 369], have been vigorously formulated by DeRobertis [121], with recent electron micrographs suggesting interchange of particulates between nucleus and cytoplasm in a mechanism perhaps parallel to that discovered by Palade for the transport of droplets across cell membranes [271]. Other evidence of mass transfer from nucleus to cytoplasm has appeared [183, 222, 288]. Nuclear volume has also been correlated with secretion [357]. The functional roles for the chromosomes that are implied in all of the foregoing also enjoy many specific illustrations, such as inverse proportions between ploidy and the numbers of cells in embryos [29, 143] and relations between ploidy and transplantability of tumors [172, 316]. Chromosomal functions in the nuclear abnormalities of cancer cells have been extensively studied. The abnormalities of chromosomes and of mitosis immediately after paintings of mouse skin *in vivo* emphasize the need for understanding the indirect relationships of nucleus and cytoplasm, as perhaps through the nucleolus [34]. Interest in the nucleolus, raised by Caspersson's earlier studies of ultraviolet absorption, has recently been extended by indications of metabolic participation, including RNA accumulation complementing DNA loss from the chromocenter [144], by the cited emission of nucleolar material into the cytoplasm, and by the persistence of nucleoli in certain circumstances, including the division of some cancer cells [33, 35]. (A recent report notes a specific effect of cobalt on nucleolar persistence [173].) From all such developments it is clear that understanding of nuclear physiology and especially of intranuclear physiology is just being opened (see 336 for a biochemical perspective).

Important gains are now being made in studies of the nuclear role in development by means of *experimentally enucleated cells,* prepared by surgery [57] or by centrifugation of favorable material. Recoveries on renucleation after three days, reported in the pioneer work of Comandon and DeFonbrune [89], emphasized the normalness of the surviving cytoplasm; but interraction of nucleus and cytoplasm was also shown by the inability of fresh nuclei to revive enucleated cytoplasm after six days. Many activities are maintained for days after enucleation [243]; these include some cell movements, irritability, ingestion and growth, formation of cellulose plant cell walls, maintenance of permeabilities [293], of biopotentials, photosynthesis, and production of fat and tannic acid. Partial regeneration may be seen in enucleated *Acetabularia* [167], and some other evidences of development independent of the nucleus have been reported [143, 170, 364, 369]. According to recent developmental studies nuclei and cytoplasm of different species (and even orders!) cooperate in cleavage and blastula formation, but a sudden development of specificity of the nucleo-cytoplasmic interaction occurs at gastrulation [58], although at least some of the nuclei within a given organism (*Rana pipiens*) are not irreversibly differentiated by late gastrula stage [211]. Such transplantation studies have also been made with triton eggs [220]. Nuclear damage may be promptly fatal to a cell; for example, the puncture of the nucleus of cultured uninucleate fibroblasts was found fatal, while puncture of one nucleus of a binucleate fibroblast had striking local effects but did not result in destruction of the cell [75, 76]. Enucleation of amoebae may not have immediately grave consequences beyond loss of reproductive capacity [98, 99]; and transfers of nuclei between species of amoebae have suggested a direct control of nuclear size by cytoplasm, and of cytoplasmic form by the nucleus. Both short-term and longer-term roles for the nucleus were clear in this special system. Recent studies of relations of the nucleus to protein synthesis exemplify the promise of such work: that protein synthesis is independent of the nucleus was first suggested by equal capacities of nucleate and anucleate segments of *Acetabularia* to incorporate CO_2 into protein carboxyl groups [51], but more recently a strong influence of the nucleus on the maintenance of protein in this alga was seen from a much greater drop in the ratio of protein-N to non-protein-N for anucleate segments [160]. Other recent data [248] compare uptakes and incorporations of methionine by whole amoebae and by cut, half amoebae (nucleate and anucleate); immediate and striking impairment of protein synthesis in the anucleate half supports a direct association of some protein-synthetic mechanisms with the nucleus. A nuclear role in RNA maintenance was also recently indicated for *Amoeba proteus* (203) and rapid nuclear uptake of orotic acid is favored by the presence of the nucleus in *Acetabularia* [52]. Such work is thus promising despite technical difficulties and uncertainties of interpretations [50].

Mitosis is perhaps the most dramatic nuclear function. It was described seventy-five years ago in living cells [149, 319, 342] (classical figures were recently reproduced [201]), and recent descriptions based on newer techniques have appeared in several books [123, 322, 371] and reviews [200, 244, 352, 361]. The division cycle occupies about half the intermitotic time, and prophase accounts for most of the mitotic time. The cinematographic records of cell division merit further careful study. Detailed studies have provided velocities of anaphase movement, in the order of several microns per minute, and such data will be helpful, as noted, in advancing analytical descriptions of mitosis [199, 202, 297]. Beautiful still and moving picture studies by polarization microscopy [198] have helped remove all doubt of the reality of traction fibers, also made clear by the proximal stretching of displaced chromosomes in centrifuged cells [330]. Fibrous protein arrays in the spindle, clearly indicated by polarization studies of living material [194, 198, 277, 320, 346], and their change in birefringence in the course of mitosis have also been described, and the mitotic apparatus has recently been isolated [244, 246]. Recent studies with glycerin-extracted "cell models" promise insight into the mechanisms of anaphase movement [178] and of cytokinesis [179]. An active role for the centromere was indicated in the early descriptions of the familiar phases of mitosis, with centromeric attachment of chromosomal fibers. This role has recently been verified

[356] by a virtual microdissection with a fine beam of ultraviolet light which, when focused on the centromeric region, blocked the poleward progression of the chromosomes. Other such studies promise further insight into mitotic mechanisms [41]. Thus, in experiments using microbeams of protons or ultraviolet light upon cultured cells from the adult newt heart [40], the indicated specific directional role of the kinetochore in chromosomal movement has been verified, along with the specificity of radiation-induced chromosomal bridging; the spindle and the cytoplasm have proved resistant to protons alone, but irradiation of cytoplasm with an ultraviolet microbeam can cause the spindle to disappear, dispersing the chromosomes. Carlson [71] has recently reported detailed microdissection studies, including an indication of chromosomal movements unaffected by bodily rotation of the spindle during anaphase. Other recent developments include detailed studies in tissue cultures [46] and descriptions of living meiotic and mitotic chromosomes by phase-contrast microscopy [82, 84]. Those have included beautiful documentation of chromosomal movements and attachment of the centromere [cf. 348] and have confirmed features such as the doubleness in prophase, major coils, the pairing of chromatids, chiasma formation, ring formation, achromatic figures with centrioles, telophase central spindle, and the bands of living salivary gland chromosomes. Such confirmations in living material of the structures seen in fixed, stained preparations are very reassuring, and aid in interpreting the cinematographic records. Recent studies of distribution of nucleolar material during mitosis [123, 142] emphasize the functional import of such cytological work. Synchronous divisions attained with some materials [318] may bring incisive developments with microbial forms and perhaps even with higher forms. The descriptive and structural studies are being paralleled by studies of lipide metabolism [206], of nucleoprotein distribution [200], of the control of mitosis through energy relations [63], of mitochondrial behavior [150], and of cyclic cytoplasmic changes during mitosis [194]. These emphasize that the whole cell is involved in mitosis.

Such studies of chromosomal constitution and function appear clearly to promise understanding of cell division, so basic in general biology and in cancer.

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5. *Studies on Mitosis in Purine-Treated Tissue Cultures*[1]

BY JOHN J. BIESELE

TISSUE CULTURE is proving its value as an aid to the understanding of mechanisms of cell division. This is especially true for mitosis in the mammalian cell. With tissue cultures, living cells of man or mouse can be observed during all phases of mitosis, and the direct effects of chemical agents applied to the cultures can be studied. Cinematographic records can be obtained with time-lapse photography. Altering the normal operation of cell division by chemical treatment, especially with analogues of normal metabolites, is a promising method of investigating the physiology and biochemistry of mitosis. It is also a fruitful technique with which to search for differences between cell types, or between normal and neoplastic cells. In this manner, studies of mitosis in chemically treated tissue cultures can contribute to the experimental chemotherapy of cancer.

Apart from tissue culture, cell division in mammalian tissues has been studied principally in fixed and stained sections, smears, and squashes. Studies of chemical effects on mitosis in such material are subject to intervention of the whole organism and its mechanisms of detoxification, localization, excretion, and adaptation. Studies of chemical effects on cell division are also carried out with less restriction in interpretation on developing eggs of marine invertebrates and on plant tissues, especially root tips of bulbs or seedlings. For those interested in the chemotherapy of cancer in man, studies on tissue cultures of mammalian neoplasms appear more likely a priori to produce useful information than do experiments with marine eggs or plant tissues. However, so far as the physiology of mitosis is concerned, different materials

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may possess unique advantages for the elucidation of particular aspects of the problem [46]. In what follows, although chief attention is paid to research with mammalian and avian tissue cultures, there is no intention of overlooking results of investigations with other materials. Firket [36] considers tissue culture the best material with which to start study of a mitotic poison because of the dynamic approach it permits. However, he would extend the study to segmenting eggs, plant material, and tumors in the animal in order to clarify certain points or to test hypotheses.

Mitosis, as the culmination of cell growth, depends on the synthesis of new protoplasm. This synthesis ordinarily occurs in the interphase before the cell division in question takes place. Desoxyribonucleic acid of the chromosomes, for example, is doubled in amount in the interphase before division, and very little if any change in the DNA quantity occurs during mitosis, according to Swift [85], and Howard and Pelc [44]. Walker and Yates [88] have shown that the amount of nuclear material absorbing at 265 mμ in the ultraviolet gradually increases during the interphasic progression from telophase to prophase in cultures of a variety of chick, amphibian, and mammalian tissues. The absorbing material probably includes purine- and pyrimidine-containing precursors of nucleic acids, as well as DNA, which is doubled by prophase and undergoes no further synthesis during that stage, according to measurements of Feulgen staining [87], or during telophase, as Deeley, *et al.* [26] find with a crushing technique. The investigations of Davidson and Leslie [24] on chick embryo heart cultures suggest that DNA synthesis is preceded by synthesis of cellular protein and phospholipide. According to Howard and Pelc [45], doubling of the protein portion of chromosomes perhaps occurs at a time in interphase different from that of DNA doubling. Uncertainty about the time of protein synthesis in the nucleus will probably be eliminated by new microscopic techniques that determine the dry mass of cytological objects [25].

Hence it appears reasonably certain that in tissue cultures the cells are dividing not on reserves but largely on materials synthesized anew during each division cycle. Although this conclusion may hold for the individual cell, it may not be true for the culture as a whole; Fischer, Laser, and Meyer [37] pointed out that carcinoma cells in culture may live and multiply for a long period at the expense of substances liberated from dying sister-cells.

The synthesis of DNA in preparation for mitosis may be found more extensive than anticipated a priori. Thus Stevens, *et al.* [82] with rat intestine and regenerating liver, Barnum, *et al.* [2] with a transplanted mammary carcinoma, and Daoust, *et al.* [22] with lung all find radiophosphate uptake into DNA to be of high enough a value to indicate a lability of most of the phosphate in chromosomal nucleic acid in each mitotic cycle, as though the desoxyribonucleic acid were torn down and rebuilt at least in part. In other words, DNA synthesis for mitosis cannot be considered to be simply a manufacture of enough new DNA, as on a template, for one daughter cell. Rather, the template itself, if such it is, is at least in part built anew in the process of being reduplicated.

Other material than that of the chromosomes must also be synthesized, and energy for the syntheses must be provided. In a study of epidermal mitosis in the mouse, Bullough and Johnson [15] have found that an interruption in the supply of energy to the tissue, as occasioned by lack of oxygen or of reserve carbohydrate, or by poisoning of respiration or glycolysis, prevents cells from entering prophase, although cells already in division may be unaffected. The mitotic machinery, or the achromatic apparatus, must be formed and be provided with what it needs for functioning. In other words, cells must be metabolically active in order to be able to divide. Lettré [64] would stress the importance of an adequate reserve of adenosine triphosphate, supplied by the cell's metabolism, to enable the cell to carry out mitosis successfully. Waymouth [47] has stated the axiomatic nature of the dependence of cellular proliferation upon adequate nutrition.

It is evident, therefore, that all sorts of agents that interfere in metabolism or render deficient a cell's nutrient supply may exert effects on mitosis. Such considerations have led various workers with mitotic inhibitors to urge that the term "mitotic poison" be restricted to agents that interfere in definite stages of mitosis [64]. Ludford [69] finds the term "mitotic poison" objectionable, because it implies that mitotic poisons are a special class of substances. Since almost any substance can influence mitosis adversely (if only to stop it), given the appropriate conditions, especially in tissue culture—Ludford [69] would prefer to state that a particular agent exerts a "mitotic poisoning action" of a certain form under specified conditions. The chief value of mitotic poisons in the elucidation of mitotic processes may lie in the enlightenment that comes with the successful blocking of certain mitotic poisons with particular physiological substances. We are thus provided with clues to the roles played by these normal physiological substances in cell division or in maintenance of that healthy basis of cell metabolism from which proliferation arises. This is a field that promises great productivity in the next few years with the upsurge of interest in chemotherapeutic mechanisms.

1. 2-Substituted Adenines and Unsubstituted Purine

For mitotic poisons to be of much value in the experimental chemotherapy of cancer, they should exert a certain selectivity of action on neoplastic cells. Bucher [14] lamented that most of the mitotic poisons studied up to 1951 had the same effects on normal cells as on tumor cells. However, the possibility that differences in nucleic acid metabolism between normal and neoplastic cells [16] might render the neoplastic cells more susceptible to attack by antagonists of nucleic acid metabolism [51] has been explored in a great number of investigations in experimental cancer chemotherapy [77]. It was early shown that the tumor inhibitor, 8-azaguanine, had a selective inhibitory effect on mitosis in certain tumor cells [78]. Reviewing the effects of some purine antagonists and some "radiomimetic" poisons such as the mustards in cancer chemotherapy, Dustin [31] concluded that the nucleoprotein metabolism was somehow altered from normal in cancerous cells. Dustin [30] had earlier found that 2,6-diaminopurine has the same effects as antifolic acids on intestinal mitoses in the mouse: mitotic arrest and the appearance of aberrant mitotic figures with damaged chromosomes.

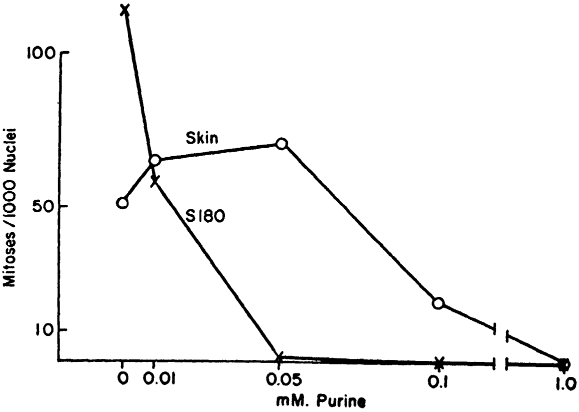
Our own work was first centered on 2,6-diaminopurine and other 2-substituted adenines [8, 10]. In this, as in later work, we have treated tissue cultures of embryonic and neoplastic mouse and human cells with analogues of the constituent parts of nucleic acids and with analogues of amino acids, vitamins, and other physiologically important substances, in attempts to capitalize on whatever metabolic differences there might be between neoplastic and non-neoplastic cells. Thus we have sought a selective inhibition or destruction of the neoplastic cells. This selective inhibition may be manifested in differential effects on mitosis, often at concentrations below those causing gross cellular damage [5].

Our cultures are planted in roller tubes, usually on coverslips inserted into the tubes. In lieu of a perfectly defined synthetic medium, we have made use of a classical medium of balanced salt solution, serum, and embryo extract, as well as a chicken plasma clot. We have used short-term cultures of embryonic tissues—skin, for the most part—and neoplastic tissues—chiefly sarcomas—fresh from the mouse. (A word of caution may be inserted here. Lettré [65] carries chick embryo fibroblasts through 15 to 20 passages in culture before using them for experimentation; and Murray, Peterson, Hirschberg, and Pool [71] carry human glioblastoma cultures for six to eight weeks before using them.)

A day of incubation is followed by a day of exposure to the agent added to the medium, and then the living cultures are examined under the microscope, in part with oil-immersion phase-contrast optics. Quantitative determinations of mitotic incidence and aberrations are made in the zones of outgrowth of cultures fixed and stained with the Feulgen reaction.

Although mouse sarcoma 180 cells appear to be some twenty times more sensitive than embryonic mouse fibroblasts to the grossly damaging effects of 2,6-diaminopurine in tissue cultures [10], mitosis is readily inhibited in cultures of both tissues by the agent near 0.1 mM [5]. In agreement with the near lack of differential in mitotic inhibition with 2,6-diaminopurine, treatment of sarcoma 180 in mice with the agent is ineffective because the minimum effective dose is at a level toxic to the animal [83].

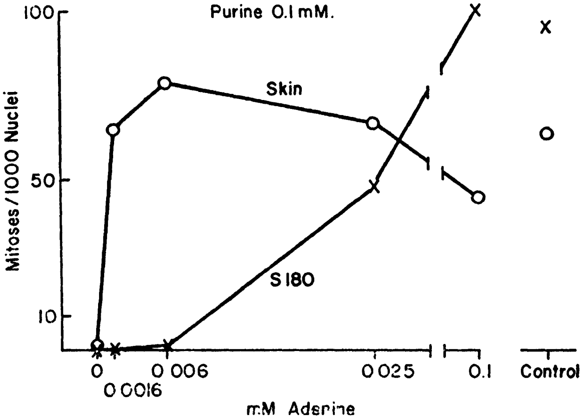
A number of adenines substituted or changed at the 2-position [3, 8], produce effects in tissue culture resembling those of 2,6-diaminopurine. To a certain extent, unsubstituted purine itself can be included in this group [12]. The mitotic inhibition it causes in tissue culture is illustrated in Text Figure A. There is a difference in the inhibition between sarcoma 180 cultures, which are more readily affected, and cultures of embryonic



TEXT FIGURE A. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 48 hours with various concentrations of unsubstituted purine.

mouse skin. There are no mitoses in cultures of either tissue at 1.0 mM purine, a few in skin cultures only at 0.1 mM purine, and many mitoses in both tissues at 0.01 mM purine.

The difference in responses of the tissues may be related to relative ease of blocking with adenine, as shown in Text Figure B. Despite the presence of 0.1 mM unsubstituted purine, adenine over the tested concentration range of 0.1 to 0.0016 mM keeps skin cultures in approximately control condition. Sarcoma 180 cells are not well protected against 0.1 mM purine by less than equimolar adenine.



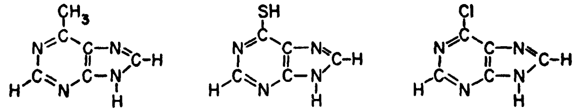
TEXT FIGURE B. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 48 hours with 0.1 mM purine plus various concentrations of adenine.

Guanine, hypoxanthine, and xanthine at concentrations up to 2.0 mM have little effectiveness, if any, in blocking the toxicity of 0.1 mM purine in tissue culture, but 4-amino-5-imidazolecarboxamide at 1.0 mM does provide moderate protection to sarcoma 180 cells [12]. Similar protection with this imidazole is observed against 2,6-diaminopurine and 2-azaadenine. It will be recalled that the ribotide of 4-amino-5-imidazolecarboxamide is an intermediate in the biosynthesis of nucleic acid purines [42]. This suggests that some of the toxicity of the 2-substituted adenines and unsubstituted purine may spring from interference in nucleic acid metabolism. Purine is also readily blocked by adenosine-3′-phosphate, but the most effective blockers of the very toxic related nucleoside, purine riboside, are adenosine-5′-phosphate and compounds with its configuration, such as diphosphopyridine nucleotide and adenosine triphosphate [12].

In studying the pharmacology of a number of purine analogues, Philips and co-workers [74] have found such a diversity of effects in the whole animal that it is likely no unitary hypothesis of their mechanism of action based on interference in normal purine or nucleic acid metabolism can prove completely satisfactory. Some of these analogues were the 6-substituted purines considered in the following section.

2. *6-Substituted Purines*

The 6-substituted purines make a group of considerable current interest. Text Figure C gives structural formulas of several of these adenine analogues in which the 6-amino group of adenine is replaced by other groups: 6-methylpurine, 6-mercaptopurine, and 6-chloropurine.[2]



6-METHYLPURINE 6-MERCAPTOPURINE 6-CHLOROPURINE

TEXT FIGURE C. Structural formulas of 6-methylpurine, 6-mercaptopurine, and 6-chloropurine.

6-Mercaptopurine has shown curative effects against sarcoma 180 in the mouse [18]. The tumor-inhibiting properties of the related compounds, thioguanine, 6-chloropurine, 6-methylpurine, and purine, have also been studied [17]. The usefulness of 6-methylpurine is limited by its high toxicity to the host.

Neither 6-mercaptopurine nor 6-chloropurine appears very toxic to mouse cells in tissue culture, but each causes differential partial inhibition of mitosis in cultures of mouse sarcoma 180. This effect has been described for 6-mercaptopurine [6] and is illustrated here in Text Figure D.

Each entry is the mean of counts, on 2 to 4 cultures, of mitoses among 1,000 nuclei in the zone of outgrowth. There is a decreased mitotic incidence at higher concentrations. This decrease is much more marked

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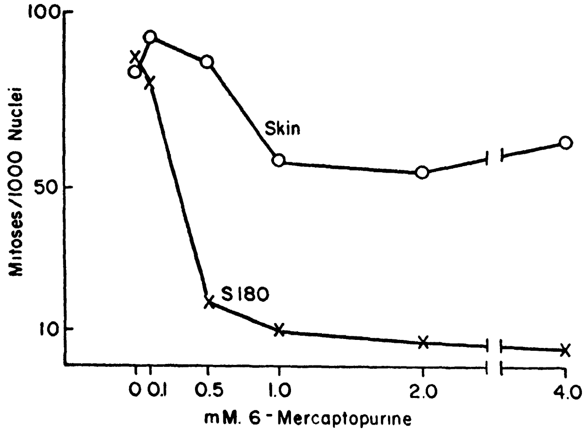
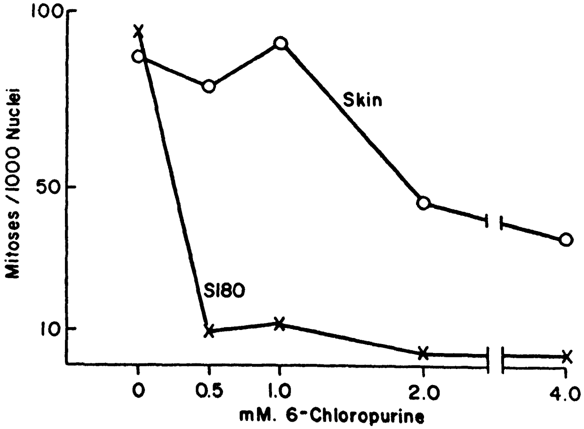
[2] The author is greatly indebted to various investigators for samples of the agents used in the present study: to Dr. G. H. Hitchings and Miss G. B. Elion of the Wellcome Research Laboratories for 6-mercaptopurine, 6-methylpurine, and thioguanine; to Dr. A. Bendich, Dr. P. J. Russell, Jr., and Dr. J. J. Fox of the Sloan-Kettering Institute for 6-chloropurine and purine; and to Dr. G. B. Brown of the Sloan-Kettering Institute for purine riboside.



TEXT FIGURE D. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with various concentrations of 6-mercaptopurine.

in the sarcoma cultures than in the embryonic skin cultures. Text Figure E presents similar results with 6-chloropurine.

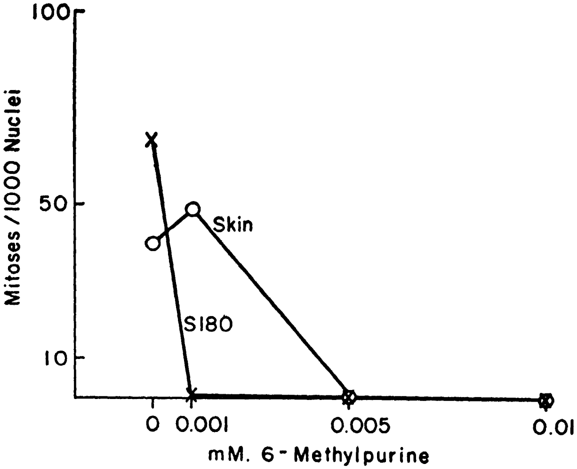
Results with 6-methylpurine are different. In Text Figure F it is seen that this far more toxic agent permits no mitosis at concentrations of 0.005 mM and above.



TEXT FIGURE E. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with various concentrations of 6-chloropurine.

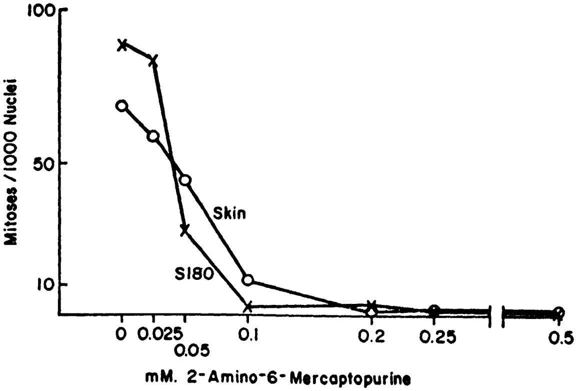
The guanine analogue, 2-amino-6-mercaptopurine or thioguanine, is of intermediate toxicity. According to Text Figure G, this compound has essentially no differential effect on sarcoma cell division.

It is of considerable interest that the order of effectiveness of these several purines in inhibiting sarcoma 180 mitosis in tissue culture matches fairly closely the order of toxicity of these agents for the intact mouse [74].



TEXT FIGURE F. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with various concentrations of 6-methylpurine.

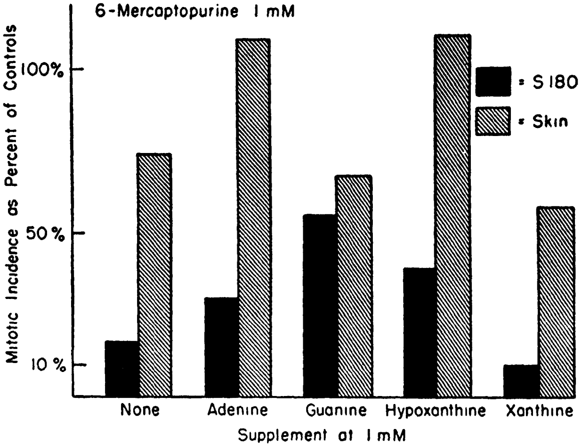
As was shown above with unsubstituted purine and adenine, the way in which a given agent exerts its effects in tissue culture can be studied to some extent with antagonism tests, in which normal metabolites presumed analogous to the possible antimetabolite are added together with it. Blocking experiments with physiological agents against the partial mitotic inhibition caused by 6-mercaptopurine bring out certain dif-



TEXT FIGURE G. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with various concentrations of thioguanine.

ferences between embryonic skin cells and sarcoma 180 cells. Some of the following data have been presented in tabular form [6].

Text Figure H shows that no physiological purine at equimolar concentration completely blocks 1.0 mM 6-mercaptopurine for sarcoma 180 cells, but guanine and hypoxanthine are most effective. Equimolar adenine and hypoxanthine are each fully protective to embryonic skin cells.

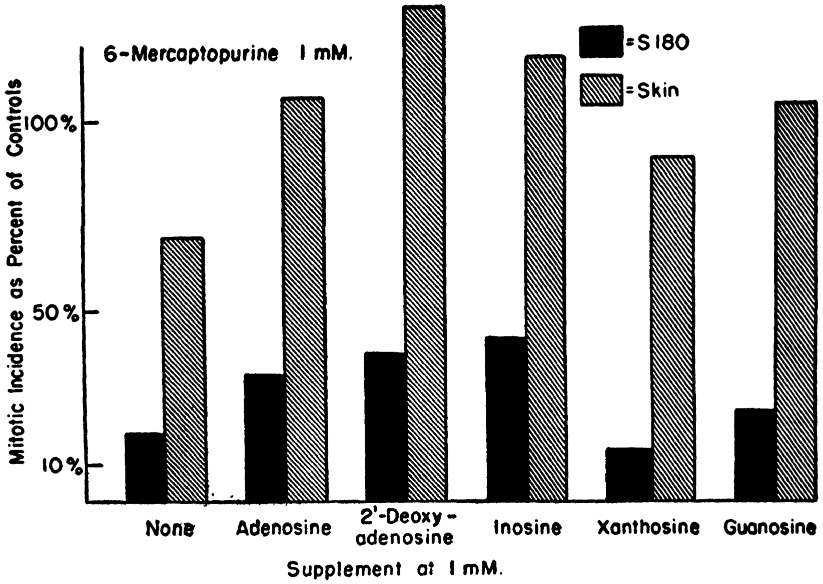


TEXT FIGURE H. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with 1 mM 6-mercaptopurine plus equimolar doses of physiological purines, referred to control incidence as 100 per cent.

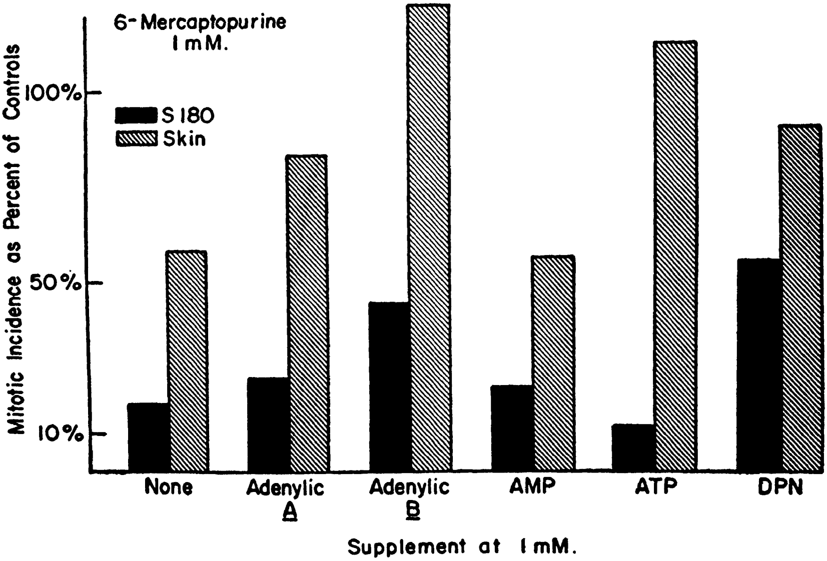
Among the purine nucleosides tested (Text Fig. I), inosine is the most effective blocker, although weak, of 6-mercaptopurine for sarcoma 180 cells. Embryo skin cells exhibit normal mitotic incidence in the presence of adenosine, inosine, or guanosine.

Among the nucleotides tested (Text Fig. J), various adenosine phosphates with the exception of adenosine-5′-phosphate are effective blockers for embryonic skin cells but relatively poor protectors of sarcoma 180 cells against 6-mercaptopurine. Diphosphopyridine nucleotide also falls into this pattern. The result seen with adenosine triphosphate is also obtained in antagonism tests with other toxic purines, such as unsubstituted purine and 2,6-diaminopurine, namely effective blocking of mitotic inhibition in embryo skin cultures but essentially no protection of sarcoma 180 cells.

In summary, a number of adenine-containing metabolites at equimolar concentration block 6-mercaptopurine inhibition of embryo skin mitosis in culture, but none of these substances is an outstanding antagonist for sarcoma 180 cells.



TEXT FIGURE I. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with 1 mM 6-mercaptopurine plus equimolar doses of various nucleosides, referred to control incidence as 100 per cent.



TEXT FIGURE J. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with 1 mM 6-mercaptopurine plus equimolar doses of various nucleotides, referred to control incidence as 100 per cent.

There are still other substances of physiological importance containing purine moieties. One of these is coenzyme A, which is involved in biological acetylations or 2-carbon transfers [67]. Coenzyme A has a salutary effect on cultured cells and prolongs their life in incomplete media [63]. In repeated experiments with different preparations of coenzyme A from several commercial sources, the mitotic inhibition caused by 1.0 mM 6-mercaptopurine was prevented in sarcoma 180 cells by 0.2 or 0.02 mM coenzyme A. Skin cells are protected by the higher concentration but not by the lower. Hence coenzyme A presented the reverse of the protection picture seen with the other adenine metabolites [6, 7].

In separate tests it was found that the results obtained could not be explained on the basis of a contamination of the coenzyme A specimen with glutathione, because glutathione is only about one per cent as effective as coenzyme A in blocking 6-mercaptopurine. Results of an experiment are given in Table 1.

TABLE 1. Effect of 6-Mercaptopurine and Glutathione on Mitotic Incidence in Tissue Cultures

Concentration		*Mitoses per 1,000 Nuclei*			
6-Mercaptopurine	*Glutathione*	*Sarcoma 180*		*Embryo Skin*	
mM	mM	*Mean*	*Standard Deviation*	*Mean*	*Standard Deviation*
—	—	114.0	29.0	69.5	3.5
1	—	24.0	1.0	57.0	7.5
1	1	27.5	11.5	34.5	2.5
1	10	102.0	1.0	57.7	5.4
—	10	120.0	27.0	63.0	2.0

Coenzyme A and 6-mercaptopurine have antagonistic effects in tissue culture with respect not only to mitotic incidence but also to mitochondrial morphology and to lipogenesis [7]. Mitochondria are shorter, thinner, and more numerous in fibroblasts treated with 1.0 mM 6-mercaptopurine, perhaps because of fragmentation. Coenzyme A prevents this change, and, alone, 0.2 mM coenzyme A makes for longer and less numerous mitochondria. Cultures treated with 6-mercaptopurine show less cytoplasmic lipid than controls, while those treated with coenzyme A have larger cytoplasmic accumulations of lipid droplets.

In view of these considerations, it may be that mitotic suppression by

6-mercaptopurine is the result, at least in part, of its action as an antimetabolite of coenzyme A. 6-Mercaptopurine might thereby interfere with the introduction of 2-carbon fragments from glycolysis into the respiratory cycle in the mitochondria. Energy production through the tricarboxylic acid cycle is necessary for mitosis of the mouse ear epidermis [15]. The effect of 6-mercaptopurine on lipogenesis in vitro is perhaps also the result of interference with a mitochondrial function of coenzyme A, participation in the biosynthesis of fatty acids [7].

Coenzyme A has not proved so effective against other analogues of physiological purines as against 6-mercaptopurine. For example, thioguanine-induced mitotic inhibition is not so readily blocked by coenzyme A, as seen in Table 2.

TABLE 2. Effect of Thioguanine and Coenzyme A on Mitotic Incidence in Tissue Cultures

Concentration		*Mitoses per 1,000 Nuclei*	
Thioguanine mM	*Coenzyme A* mM	*Sarcoma 180* *Mean*	*Embryo Skin* *Mean*
—	—	149	86
0.1	—	19	16
0.1	0.2	62	73
0.1	0.02	16	13

There is a related agent which also makes for longer mitochondria. This is α-lipoic acid,[3] which is responsible for transferring acetyl from pyruvate to coenzyme A, perhaps in combination with thiamin pyrophosphate or cocarboxylase [75]. Fibroblasts treated with α-lipoic acid show mitochondria enlarged somewhat over the control, and the cultures are fatty. However, the ability of α-lipoic acid to prevent mitochondrial fragmentation under 6-mercaptopurine treatment is inferior to that of coenzyme A.

The mitotic incidence in an experiment to test the antagonism between α-lipoic acid and 6-mercaptopurine is given in Table 3. Unlike coenzyme A, lipoic acid does not produce a dramatic release from the mitotic inhibition caused by 6-mercaptopurine. It even seems to accentuate the inhibition of embryonic skin cells. However, α-lipoic acid im-

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[3] The author is indebted to Dr. L. J. Reed of the University of Texas for a sample of *DL*- α-lipoic acid.

TABLE 3. Effect of 6-Mercaptopurine and *DL*-α-Lipoic Acid on Mitotic Incidence in Tissue Cultures

Concentration		*Mitoses per 1,000 Nuclei as Percentage of Controls*	
6-Mercaptopurine mM	*α-Lipoic Acid* mM	*Sarcoma 180* *Mean %*	*Embryo Skin* *Mean %*
—	—	100	100
1	—	36	92
1	0.2	42	62
—	0.2	134	141

proves the effect of coenzyme A against the 6-mercaptopurine-induced inhibition of sarcoma cell mitoses, according to the experiment of Table 4.

The effect of α-lipoic acid in potentiating the mitotic inhibition of embryonic skin cells by 6-mercaptopurine is paralleled by a similar effect of insulin (Table 5). In this experiment the decrease in mitotic incidence in cultures treated with both insulin and 6-mercaptopurine, as compared with those treated with 6-mercaptopurine alone, is just on the borderline (92 per cent level) of statistical significance for the skin cultures, and slightly below it (86 per cent level) for the sarcoma cultures. Insulin alone is without effect.

In a second experiment with insulin, as seen in Table 6, insulin plus 6-mercaptopurine caused a decrease in mitotic incidence from 6-mercaptopurine alone statistically significant on the 95 per cent level of confidence for embryonic skin.

That insulin and α-lipoic acid should have a similar depressing effect on mitotic incidence in embryonic skin cells in the presence of 6-mercaptopurine may not be pure coincidence. Insulin may act to increase glu-

TABLE 4. Effect of 6-Mercaptopurine, *DL*-α-Lipoic Acid, and Coenzyme A on Mitotic Incidence in Tissue Cultures

Concentration			*Mitoses per 1,000 Nuclei*	
6MP mM	*Lipoic* mM	*CoA* mM	*Sarcoma 180* *Mean*	*Embryo Skin* *Mean*
—	—	—	104	58
1	—	—	27	47
1	0.2	—	45	40
1	—	0.2	85	66
1	0.2	0.2	147	62

cose consumption not only by activation of hexokinase or by some effect on cell membrane permeability, but also, according to Foa, *et al.* [39], by preventing the dephosphorylation of thiamin pyrophosphate. α-Lipoic acid may in some organisms form a coenzyme in combination with thiamin pyrophosphate [75]. Both agents appear to be of importance in lipogenesis. But why they should increase mitotic inhibition from purine analogues is not readily apparent. Insulin has been found by Paul and Leslie [73] to increase the rate of mitosis, the consumption of glucose and oxygen, and the synthesis of nucleic acid and protein in tissue cultures. Lu and Winnick [68] report that insulin increases the incorporation of exogenous isotopic adenine into nucleic acid of tissue cultures. Presumably insulin places such a demand on the synthetic machinery that endogenous purine from biosynthesis does not suffice, and purine from without is used. Radioautographic techniques have demonstrated in our tissue cultures the uptake of adenine-2-C^{14} into the chromosomes of embryonic skin cells [38].

TABLE 5. Effect of 6-Mercaptopurine and Insulin on Mitotic Incidence in Tissue Cultures of Mouse Sarcoma 180 and Embryo Skin

Concentration		*Mitoses per 1,000 Nuclei*			
6-Mercaptopurine	*Insulin*	*Sarcoma 180*		*Embryo Skin*	
mM	unit/ml	*Mean*	*Standard Deviation*	*Mean*	*Standard Deviation*
—	—	104.8	4.7	87.3	7.7
1	—	22.3	4.0	39.5	9.4
1	1	14.5	2.3	18.3	3.0
—	1	91.5	8.2	91.4	18.3

TABLE 6. Effect of 6-Mercaptopurine and Insulin on Mitotic Incidence in Mouse Embryo Skin Cultures

Concentration		*Mitoses per 1,000 Nuclei*	
6 Mercaptopurine mM	*Insulin* unit/ml	*Mean*	*Standard Deviation*
—	—	108.3	36.0
1	—	56.0	5.5
1	1	39.3	1.8
—	1	97.3	15.1

It is possible that an incorporation of 6-mercaptopurine into nucleic acids in place of adenine may account for some of the toxicity and mitotic inhibition exhibited by this analogue in tissue cultures, especially if the culture conditions lead to an increased uptake of exogenous purine.

It is generally held that 6-mercaptopurine interferes with nucleic acid metabolism and that its toxicity may be counteracted by a number of physiological purines, nucleosides, and nucleotides [33, 41]. It is reported that 6-mercaptopurine inhibits *de novo* synthesis of nucleic acid [79] or may be itself incorporated into nucleic acid after injection into mice [32].

An additional indication that an interference in nucleic acid metabolism may be in part responsible for the enhancement of mitotic inhibition from 6-mercaptopurine in tissue cultures in the presence of insulin or α-lipoic acid is given by the increased frequency of mitoses with damaged chromosomes under such conditions.

3. Chromosomal Damage

The chromosomal damage from purines in tissue culture is illustrated in Figures 48 to 53. An example of the failure of chromosomes to be included in the metaphase plate is given in Figure 48. This delayed metaphase was in an embryonic skin culture treated with 1 mM thioguanine plus 1 mM adenosine triphosphate. Such an aberration is presumably the result of centromere defect, for similar failure to join the metaphase plate may be induced in chromosomes irradiated with a micro-beam of ultraviolet, but only if the region of the centromere is irradiated [86]. This figure also resembles the C-mitosis caused by colchicine, or the partial inhibition of anaphase movement seen by Hughes [48] in tissue cultures treated with high concentrations of adenine, 2,6-diaminopurine, or benzimidazole.

Figure 49 is another example of failure of chromosomes to be included on the plate. It occurred in a skin culture treated with 0.0025 mM purine riboside. These laggard chromosomes are displaced to the poles somewhat in the manner described by Parmentier [72] for mitoses treated with hydroquinones or phenols.

In mitotic stages after metaphase, chromosomal damage is evident in anaphase and telophase figures with chromosomal bridges or fragments, as represented in Figure 50. This bridged anaphase with one attached fragment is from an embryonic skin culture treated with 0.05 mM thioguanine. Figure 51 shows a late telophase with one bichromatid fragment in a sarcoma 180 culture treated with thioguanine. In both Figures 50 and 51 the lagging chromosome fragments are attached by fine threads to the separating sister groups of chromosomes. In general they resemble the aberrations called pseudochiasmata by Levan and Tjio [66], thought to result from fusion in one point of four broken chromatids [19]. They are interpreted by La Cour and Rutishauser [62] as the result of subchromatid breakage and reunion induced by treatment during prophase.

When insulin is added with thioguanine to embryonic skin cultures, the percentage of postmetaphasic mitotic figures with chromosomal bridges or eroded and broken chromosomes is increased several fold over their percentage with thioguanine alone (Table 7).

TABLE 7. Effect of Thioguanine and Insulin on Incidence of Aberrant Postmetaphase Figures in Mouse Embryo Skin Cultures

Concentration		*Skin Postmetaphase Figures*	
Thioguanine mM	*Insulin* unit/ml	*Total Studied*	*% with Bridges and Breaks*
—	—	43	0
0.05	—	246	8.5
0.05	1	181	46.9

Insulin alone causes no apparent chromosomal damage in our cultures, as is seen in Table 8. This experiment also illustrates the suggestion of an increase in chromosomal damage obtained repeatedly with insulin and 6-mercaptopurine.

TABLE 8. Effect of 6-Mercaptopurine and Insulin on Incidence of Aberrant Postmetaphase Figures in Mouse Embryo Skin Cultures

Concentration		*Skin Postmetaphase Figures*	
6-Mercaptopurine mM	*Insulin* unit/ml	*Total Studied*	*% with Bridges & Breaks*
—	—	211	0.9
1	—	105	0.9
1	1	100	7.0
—	1	203	0

The potentiation of chromosomal damage from 6-mercaptopurine by α-lipoic acid is shown in Table 9. It is of interest that the additional presence of coenzyme A eliminates the tendency to form chromosomal bridges and breaks. Chromosomal damage from purine analogues in tissue culture may thus be regarded as subject to composition of the medium or in a sense dependent on the physiological condition of the cells as determined by the medium.

TABLE 9. Effect of 6-Mercaptopurine, α-Lipoic Acid, and Coenzyme A on Incidence of Aberrant Postmetaphase Figures in Mouse Skin Cultures

Concentration			*Skin Postmetaphase Figures*	
6-Mercaptopurine mM	*Lipoic* mM	*CoA* mM	*Total Studied*	*% with Bridges & Breaks*
—	—	—	387	0.8
1	—	—	279	9.7
1	0.2	—	247	24.7
1	—	0.2	232	0.9
1	0.2	0.2	164	1.8
—	0.2	—	342	1.5
—	—	0.2	259	0.8
—	0.2	0.2	177	2.3

Chromosomal damage from purines also depends on the nature of the purine itself. The three purines, 6-mercaptopurine, 6-chloropurine, and 2-amino-6-mercaptopurine, differ considerably in their effectiveness in inducing mitotic aberrations in tissue culture. One aberration often caused by 6-chloropurine in sarcoma 180 cultures, for example, is failure of chromosomes to separate into sister sets after metaphase. This aberration may be interpreted as resulting from thoroughgoing centromere damage or from spindle inactivation or from both. Figure 52 illustrates an unseparated late telophase as a restitution figure of some 10 lobes, in a sarcoma culture treated with 1 mM 6-chloropurine plus 1 mM diphosphopyridine nucleotide.

Frequencies of these postmetaphasic aberrations—failure of chromosomes to separate, and the occurrence of bridges or fragments, either broken or still attached by an eroded thread to the poleward-moving centromere—are given in Table 10.

Against a control incidence of aberrant forms among the postmetaphasic figures of 3.3 per cent for sarcoma 180 and 1.5 per cent for embryonic skin, cultures treated with 6-chloropurine show aberrations in 85 per cent of sarcoma postmetaphases and 6 per cent of embryonic skin postmetaphases. Cultures treated with thioguanine show a frequency of 44 per cent aberrant forms in sarcoma postmetaphases and 23 per cent in embryonic skin metaphases. Cultures treated with 6-mercaptopurine have incidences of mitotic aberrations nearer the control values, 17 per cent for the sarcoma, and 7 per cent for the skin.

TABLE 10. Effect of 6-Substituted Purines on Incidence of Aberrant Postmetaphase Figures, Unseparated or with Chromosome Bridges and Breaks, in Mouse Sarcoma 180 and Embryo Skin Cultures

	POSTMETAPHASES					
	Mouse Sarcoma 180			*Mouse Embryo Skin*		
	Total	*Unseparated* %	*With B & B* %	*Total*	*Unseparated* %	*With B & B* %
Control	2,553	0.04	3.3	1,930	0	1.5
6-Mercaptopurine 1 mM	265	5.3	11.3	1,031	0	7.2
Thioguanine 0.1 and 0.05 mM	195	12.8	30.8	475	4.6	18.1
6-Chloropurine 1 and 0.5 mM	79	38.0	46.8	741	0.3	5.3

Chromosomal damage from a variety of purines in plant material has been described by Kihlman [53–56]. He has shown [55] that there is a relation between lipoid-solubility of purine derivatives and their ability to effect chromosome breaks in onion root tips, apparently determined by the presence of a lipoidal layer around the nucleus in the onion root tip. Kihlman has also [56] noted an agreement between relative "solubilizing power" and chromosome-breaking proclivity of purine derivatives.

Duncan and Woods [29] have reported on chromosomal damage induced in plant materials by 5-aminouracil. They have stressed the attachment of chromosomal fragments to the main body of the chromosome through a Feulgen-negative thread. Such erosion of chromosomes has also been described by others, notably Levan and Tjio [66] for phenols. Martinez Pico and Duncan [70] have found that the chromosomal erosion from 5-aminouracil can be antagonized by cytidine sulfate. Kihlman [57], however, has expressed doubt that the breakage of chromosomes by purines results from an effect on nucleic acid synthesis, because abnormal purines that can be incorporated into nucleic acids do not necessarily cause chromosome breaks, and because there may be biochemical antagonism between certain purines and structurally unrelated compounds, among other reasons.

Despite Kihlman's reservations [57] concerning the antimetabolite hypothesis, some examples may be cited in support of the suggestion of a mutual antagonism between adenine and 2,6-diaminopurine with respect to the induction of chromosomal aberrations [9]. Unsubstituted purine in tissue culture causes an increase in the percentage of sarcoma 180 anaphases and telophases showing chromosomal bridging or fragmentation, and this percentage can be decreased by the simultaneous administration of adenine [12]. Unsubstituted purine also causes a prolongation of metaphase relative to prophase in embryonic mouse skin cultures, and adding adenine tends to counteract the effect [12].

Some cytologists would probably look on the "sticky" bridges in the anaphase of Figure 53, from a sarcoma culture treated with 0.01 mM purine, and the often attached "fragments" in mitoses in tissues treated with purines or pyrimidines, as evidence of faulty chromosomal reduplication and separation due to some sort of nucleic acid upset rather than as evidence of true structural rearrangements in chromosomes, such as are caused by ionizing radiations and by certain chemicals, including the mustards and ethylenimines, some of whose effects mimic those of radiation. Certainly the purine-damaged mitoses in tissue culture do not resemble very closely the aberrant mitoses produced by the ethylenimine-s-triazines, such as the anaphase of Figure 54, with fragment chromosomes left at the equatorial plate. Nor do the mitoses in purine-treated tissue cultures closely resemble the bridged anaphase with unattached and probably acentric fragments in Figure 55, from a skin culture treated with pyridoxine-nitrogen mustard. Similar figures are often seen with the ethylenimine triazines and the ethylene phosphoramides [4]. However, the recent work of Kihlman [57] on the similarities between chromosome breakage by 8-ethoxycaffeine and that by X-rays, including the effects of oxygen, supports the thesis that purines can cause true structural rearrangements in chromosomes. If purines do cause true structural rearrangements in our tissue cultures, it may be that postmetaphase figures with bridges and fragments produced by purines look different from aberrant mitoses in cultures treated with mustards or ethylenimines because of a greater amount of so-called "stickiness" with purine treatment. It may be of greatest importance to our understanding of "stickiness" to note that it occurred in root-tip mitoses after degradation of ribonucleoproteins by means of ribonuclease treatment [49].

4. Chromosome Breakage and Mutagenicity

It has been generally held since the classic paper by Darlington and Koller [23] on the chemical breakage of chromosomes that agents such as the mustards cause structural rearrangements in chromosomes. These seem to be of the same nature as those caused by ionizing radiations (their "secondary effect") but may result from different initial reactions, according to Koller and Casarini [61]. Chromosome breakage in tissue culture from sulfur mustard and from nitrogen mustard has been described by Fell and Allsopp [34, 35].

All chromosome-breaking chemicals are defined as mutagenic by D'Amato [21]. This may be an extreme position to take, because there does not seem to be a perfect correlation between the ability to cause gene mutations and the property of breaking chromosomes, according to the literature. We may nevertheless recall that mustards are strongly mutagenic [1] and that certain purines, notably caffeine, exhibit mutagenic properties [40]. However, the several purines tested by Hemmerly and Demerec [43] among 27 chemotherapeutic agents exhibited slight mutagenicity at best. One of these weak mutagens is 6-mercaptopurine. Although the list did not include thioguanine, it did include O-diazoacetyl-*DL*-serine,[4] or azaserine, as one of the most active mutagens for *E. coli* Demerec had ever tested. In our mouse tissue cultures, however, azaserine causes only a slight increase in frequency of postmetaphasic figures with bridges and fragments. Thus azaserine at a concentration of 0.1 to 0.01 mM caused only 5.6 per cent aberrant among 250 postmetaphases in sarcoma 180 cultures as against 3.3 per cent for the controls, and only 3.1 per cent aberrant among 751 postmetaphases in embryonic skin cultures as against 1.5 per cent in the controls. This is less than the effectiveness of 6-mercaptopurine. Mitotic incidence in the tissue cultures under a series of concentrations of azaserine is given in Text Figure

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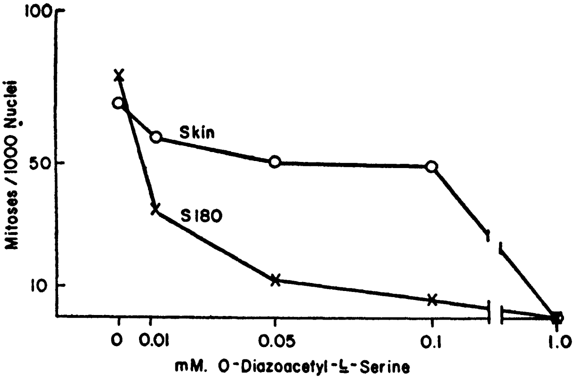
[4] The author is indebted to Parke, Davis and Company for a sample of azaserine.

K, which resembles in general that given for purine in Text Figure A. The inhibitory activity of azaserine on mouse sarcoma 180 *in vivo* has been described by Stock, *et al.* [84].



TEXT FIGURE K. Mitotic incidence in outgrowths of mouse embryonic skin and sarcoma 180 cultures treated 24 hours with various concentrations of azaserine.

Just how chemicals exert their effects on mitosis and chromosomes is an intriguing problem that has called forth a welter of hypotheses. Koller [60] has stressed the fact that chromosomes are dynamic entities within a nucleo-cytoplasmic complex, that components of their structure are exchanging with the rest of the complex and with the medium, and that chemical mutagens may act not only on the chromosomes already formed but also on those undergoing synthesis.

The hypothesis that chromosome breakage in the tumor cell arises from metabolic disturbances and may be influenced by the environment is beautifully illustrated in Koller's study [59] of a dicentric chromosome in a rat tumor. The bridge-break-fusion cycle, which could be followed in the solid tumor, did not appear in the tumor in tissue culture or in the ascitic form. This has similarities to the situation discovered by Demerec and Hanson [28]. They found that the effectiveness of a mutagen can be modified by changing the physiological state of the cell through its environment. Although manganous chloride is strongly mutagenic for resting bacteria, it has little effect on growing bacteria. A new hypothesis of mutation by Demerec [27] states that a mutagen does not affect a gene directly, but induces a change in some other cell constituent, which in turn modifies cell metabolism and thus affects the stability of the gene. Indeed, Resende [76] theorizes that spontaneous mutations and structural changes in the chromosomes may result from spontaneous changes in metabolism, or that an excess of normal substances will suffice, even in the absence of any new mutagenic agents. Much the same theory is advanced by D'Amato [20], who adds a variation on the theme in suggesting that under special physiological conditions cells might not be able to withstand the concentrations of certain metabolites normally present. This brings us close to the hypotheses that would have some of the mitotic irregularities in cancers derive from an excess of compounds (for example, adenine) released from necrotic cells.

On the other hand, deficiencies are also known to result in chromosomal abnormalities. *Tradescantia* grown with low sulfate concentrations or without magnesium shows irregular meiotic divisions [80, 81]. An excess in the midst of deficiency, or an unbalance, prevailed in Kieler's [52] cultures of chick embryo fibroblasts in dialyzed medium supplemented with aspartic acid; these cells showed chromosome breakage after an initial stimulation of mitosis. Any treatment with an effective dose of antimetabolite makes for a nutritional deficiency, as with fluorophenylalanines in tissue cultures; and these compounds also cause chromosomal disturbances [11]. Kaufmann, *et al.* [50] have noted that changes induced by irradiation in any of the constituent parts of the complex nucleoprotein that is the chromosome will modify the organization of the whole complex.

The present work contributes to the concept of the influence of the physiological state of the cell on chromosome breakage, whether "spontaneous" or induced, by the observation of the potentiating effect of insulin or α-lipoic acid on abnormal purines.

A recent investigation by Kihlman [58] is highly relevant. He finds that the chromosome-breaking activity of 8-ethoxycaffeine in onion roots is enhanced by oxygen, air, or hydrogen peroxide, but it is inhibited by cyanide or azide as cytochrome oxidase inhibitors or by dinitrophenol as an uncoupler of oxidation and phosphorylation. He interprets these observations to mean that the presence of compounds containing energy-rich phosphate bonds is necessary for chromosome breakage by 8-ethoxycaffeine. Although this interpretation runs somewhat counter to our own observation of reduced incidence of chromosomal damage when coenzyme A is added to the combination of 6-mercaptopurine and α-lipoic acid, it does agree with the general thesis of influence of the physiological state of the cell on the induction of breakage.

It thus appears that, besides the possibilities that chemicals may interfere in enzyme activity in chromosome synthesis [13], or that chemicals may interfere in the functions of nucleic acid, or that antimetabolites—for example, antipurines—may be incorporated into chromosomes in place of their normal counterparts, there is also the possibility that an unbalanced provision of raw materials for the synthesis of the complex structure of chromosomes, with their DNA, their RNA, and their various proteins and possibly other substances, may result in faulty synthesis and eventual breakage.

5. *Conclusion*

Although inhibitors of one class of compounds may exhibit family resemblances in the mitotic disturbances they cause, there may also be differences in effects between compounds within one group. With purines, differences in expression of effect may be pronounced. Even slight changes in structure of the inhibitor molecule may suffice to shift its physiological effects considerably.

A variety of metabolic processes may apparently be adversely affected by any one agent, and related agents may differ in which processes they affect more strongly. With abnormal purines, there is reason to suspect interference not only in nucleic acid metabolism but also in the metabolism of nucleotide coenzymes. In this last respect, attention is drawn to the relation between coenzyme A and 6-mercaptopurine.

Cells of different lineages may differ in their response to inhibitors, and selective inhibition of neoplastic mammalian cells is considered as a practical possibility. The response of a given cell type is in part subject to the composition of the medium and presumably to the environmentally determined physiological state of the cells.

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6. *Changes in the Desoxyribonucleoprotein Complex during the Cell Cycle*[1]

BY DAVID P. BLOCH

1. Introduction

RECENT BIOLOGICAL PROGRESS has been marked by a gradual overlapping of once separate disciplines. In the morphological sciences the trend has been toward a progressive decrease in the size and complexity of the units under investigation. Even before the advent of the electron microscope some of the most interesting problems at the cytological level have dealt with cellular structures which border on the molecular, and whose physiological significance is often best explained in chemical terms. In consequence, the means for applying the type of analytical procedures familiar to the chemist have been eagerly welcomed by the cytologist.

Biochemistry, however, has progressed in the opposite direction. The complex macromolecules involved in such processes as the synthesis of the formed elements of the cell, of great interest at present, may often be more readily defined in morphological than in chemical terms. The operation of these systems depends to a large degree upon a high level of organization compatible with the relatively unaltered structural elements of the cell. Often, moreover, reactions of interest to the biochemist may occur within perhaps only a few cells, and be obscured by the myriad processes which occur simultaneously in the tissues of which these cells make up but a small part. The classical biochemical approaches based in the main upon destruction of the tissue and extraction of its constituents suffers from limited application at these levels. Procedures which result in the maintenance of the structural elements of the cell and of the individual cells themselves, on the other hand, have become increasingly attractive.

It is more than coincidence, then, that studies of the chemical aspects

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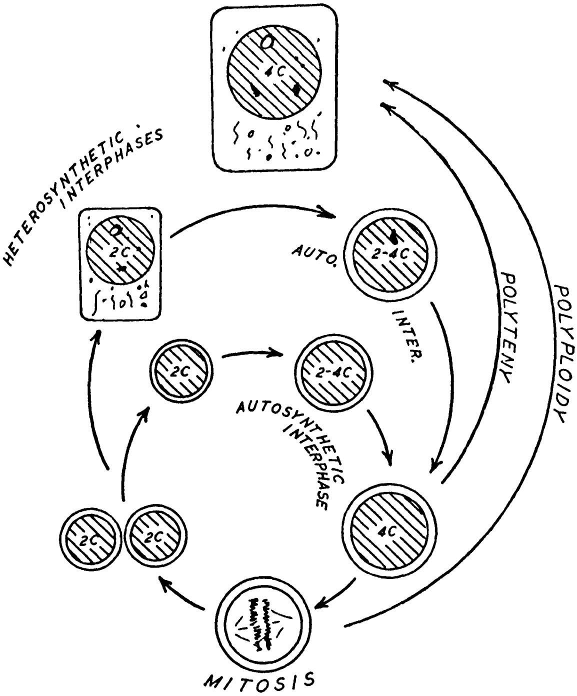
of the morphological entities of the cell have increasingly utilized procedures embodying combinations of techniques borrowed from both the biochemist and cytologist. This is exemplified by the technique of cytophotometry which enables the cytologist to study and measure the various chemical cellular constituents in the intact, though not necessarily living, cell. The advantage of this approach does not lie solely in the ability to measure very small objects. Equally important is the fact that it combines quantitative analysis with a subjective type of information which cannot be obtained other than by microscopical observation.



TEXT FIGURE A. Schematic representation of the stages of the cell cycle.

This is illustrated by consideration of one problem to which the technique of cytophotometry has proved itself singularly adapted: the relationships between chromosomal synthesis and the various stages of the cell cycle. These stages, schematically illustrated in Text Figure A, include (a) the phases of mitosis; (b) the autosynthetic interphase stage characterized by synthesis of new chromosomal material as a necessary prelude to division; and (c) the heterosynthetic stages, during which the cell operates in the specialized manner commensurate with its state of differentiation but does not directly take part in proliferative activity

[28]. Since most proliferating tissues contain a mixture of a number of cell types, in all these stages, the cytophotometric approach with its ability to single out and study individual cells has here a decided advantage over biochemical techniques, which in most cases must rely on statistical differences between tissues which contain fewer or more of any one cell stage. Even if morphological criteria by which these cell stages can be identified are lacking, cytophotometry will permit the correlation of a number of variables such as cell size, DNA content, and protein content, thereby affording a means for their identification and allowing the establishment of a pattern through which the various developmental pathways can be described.

The purpose of this chapter is to relate the changes which occur in the desoxyribonucleoprotein complex throughout the various stages of the cell cycle to the various known chemical and biological properties of these substances, and their suspected physiological functions. The discussion will of necessity draw primarily upon cytophotometric evidence in the description of these changes. This technique is still sufficiently novel so that some mention should be made of its underlying principles, possibilities, and limitations. However, the incursion here into these aspects will be rather brief. For a more extensive treatment of this and the methodology, the reader is referred to a number of recent reviews covering these facets in greater detail [ultraviolet cytophotometry: 29, 38, 39, 143; visible cytophotometry and chemical reactions of cellular constituents: 55, 142, 157, 162, 195, 197; general: 52, 54, 139, 159].

PHYSICAL BASIS OF THE CYTOPHOTOMETRIC TECHNIQUE. The cytophotometric technique is based upon determination of the relative amounts or concentrations of cellular substances by measurement of the light absorption characteristics of the native substances, or of their colored chemical combinations. The original work of Caspersson and his school dealt mainly with the qualitative aspects of the method: the identification of the cellular components by means of the shape of their absorption curves, and the localization of these substances in the cell by the characteristic absorption curves of the various cell organelles [38]. Estimation based upon the absorption spectra of naturally occurring constituents suffers from a lack of specificity, because of the difficulty in distinguishing between ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) on the one hand, and between the various protein components on the other. These methods have been in many respects superseded by Pollister's technique and its modifications

[139, 150, 164], which can take advantage of a variety of colorimetric reactions used in chemical analysis. Reactions such as the Feulgen procedure result in the specific staining of these cell components, permitting their quantitative determination.

The physical basis of the cytophotometric technique rests upon the well known Beer-Lambert laws which state that

$$E = ln\frac{I_o}{I} = kcd$$

where E is the extinction or optical density; I, intensity of the transmitted light; I_o, intensity of the incident light; k, the specific extinction coefficient; d, the distance through the absorbing substance; and c, the concentration of absorbing substance. These equations state that the extinction, or the log of the fraction of light transmitted through an absorbing substance, is proportional to the number of absorbing molecules present.

In practice, the usual procedure in the determination of the amount of chromophore, or absorbing material, in a nucleus for example, is to measure the extinction of a plug through the nucleus, treating this plug much as a cuvette in a chemical spectophotometer or colorimeter. The amount of chromophore in the entire nucleus is then calculated from the amount in the plug, and the fraction of the nucleus contained in the plug [2, 193]. Where only the comparison of a number of similarly shaped nuclei is of interest, with regard to the *relative* amounts of chromophore contained in the individual nuclei, the parameters k, c, and d can often be eliminated from the calculations. In a simple case such as the determination of relative amounts of chromosomal nucleic acid or protein in a population of interphase cells flattened by squashing, the formula is reduced to

$$S = EA = A \cdot log\frac{I_o}{I}$$

where S is the amount of stained substance and A the nuclear area. All three necessary parameters, A, I, and I_o, are easily obtained by direct measurement.

Unfortunately, the objects of interest are not always so amenable to measurement as are the flattened nuclei. Often the cells to be studied must be considered in context with their immediate surroundings, precluding squashing procedures. In such a case it is necessary to work with stained tissue sections in which nuclei will occur in a variety of shapes and sizes throughout the section. Here the main problem is one of geometry, relating the size of the plug to that of the rest of the nucleus. In this case approximations must be used, the validity of which can only be judged on the basis of common-sense application of simple geometrical formulae. For example, in a population consisting of both spherical and prolate spherical (cigar shaped) nuclei, a single formula must be used which will be applicable to all the objects measured. In this particular case

$$S = \frac{ER^2}{F} \cdot \frac{a}{b}$$

where R is the radius of the plug; F, a correction factor relating R to b [193]; and a and b, the long and short radii of the spheroid. If the results obtained by the use of such approximations are similar to those obtained with a proven model—the flattened nuclei for instance—this serves as strong presumptive evidence of their validity.

Sometimes nuclei vary in such a manner that they cannot be treated simply as problems in geometry. In dealing with irregularly shaped nuclei, or those found during mitosis with heterogeneous distribution of chromophore, other means must be applied. At present, two of the most useful are the two wave-length method [145, 150] and the scanning technique [56].

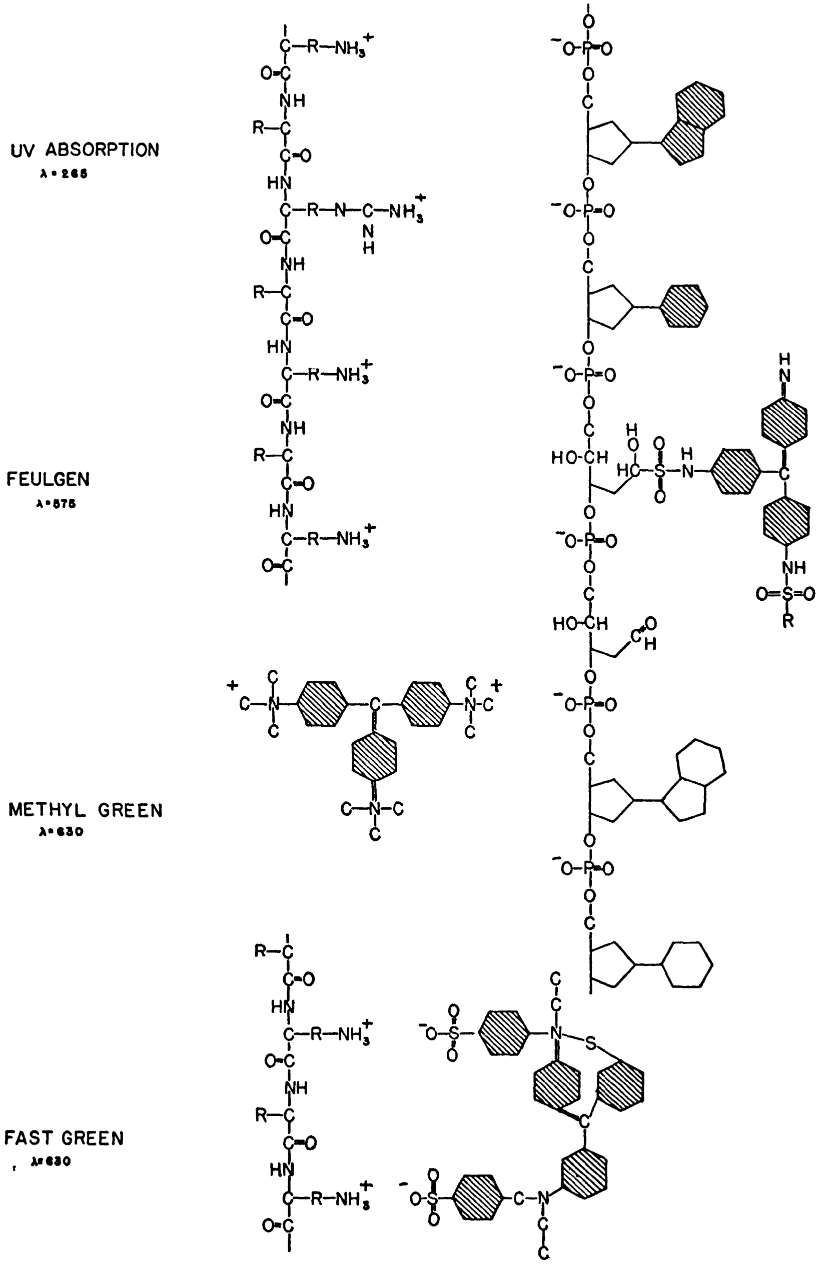
As the cells proceed through the various stages of development, changes in their staining properties may arise which reflect departure from the Beer-Lambert relations. While these laws are of prime consideration in quantitative studies, it should be stressed that if deviations therefrom, arising from chemical or physiological sources, can be controlled and correlated with a developmental process, there may be provided extremely useful information which might otherwise not be available. Examples of this will be seen in the section on chromosomal proteins.

Table 1 gives a partial list of the most common sources of error which can result in the departure from the Beer-Lambert relationship. Most of these conditions can be eliminated or minimized in practice. Some are as yet insurmountable in a number of staining techniques and serve to eliminate these from quantitative use. The limited number of chemical procedures currently available which fulfill the requirements for quantitative use imposes the most severe restriction upon the cytophotometric technique. The techniques which have met with most success in the analysis of the chromosomal components are (Text Fig. B):

TABLE 1. Some of the More Frequent Sources of Error Encountered in Cytophotometry, and Their Corrections *

Sources of Error	*Examples*	*Corrections*	*References*
CHEMICAL			
nonspecificity	UV absorption, methyl green stain	removal of source of interference	28, 70
absorbed dye varies with concentration of substrate or that of interfering substances	most routine histological stains, basic staining of DNA, acid staining of histone	removal of source of interference	8, 28
specific extinction coefficient varies with dye concentration.	metachromasy	select conditions under which k is constant	68
PHYSICAL			
error due to heterogeneous distribution of chromophore	mitotic figure	two wave length, scanning, or photographic methods	56, 145, 150
irregular geometry enclosing absorber	irregularly shaped or nonspherical nuclei	above methods, or appropriate calculations	above, or 2,193
nonspecific light loss	refractive index differences	mounting medium of correct refractive index	157
INSTRUMENTAL			
stray light	glare	use of substage objective, decreased N.A., matched refractive indices	141, 146, 164
distributional error	nonrandom nonuniform response over illuminated area of photocell	selection of proper area on photosensitive surface, focus rear lens of objective on photocell, use of ground glass or lens	156, 193

* See Moses [139] for a more complete list.



TEXT FIGURE B. Schematic representation of the chromosome, indicating naturally occurring chromophores of the nucleic acids and artificial chromophores applied during staining procedures.

ultraviolet absorption [37, 38, 70, 209, 210], methyl green staining [28, 70, 108, 161], and the Feulgen procedure [197], all of which are methods for determination of DNA; Alfert and Geschwind's alkaline fast green technique for histone [7, 8, 27]; and to a lesser extent, also, modifications of the Millon reaction [164] and naphthol yellow staining [57]. The results obtained with some of these methods are subject to variation depending upon the condition of the substrate; but this, instead of being a liability, can contribute much additional information. Judicious use of ultraviolet absorption coupled with enzymatic removal of interfering substances can be used for determination of DNA, RNA, or if applied to living cells, the total nucleotide content [210]. Methyl green staining depends upon the binding of the basic dye onto the acidic linkages of the DNA and may be used to a limited extent as an indication of the degree of polymerization of the DNA [108, 109, 116, 204], and also, of the nature of the protein-DNA linkage [3, 28]. Feulgen staining, based upon the removal of the purines by acid hydrolysis and reaction of the exposed aldehydes with Schiff reagent [60, 67, 113], is, with few possible exceptions [183, 184], relatively insensitive to qualitative variation in the desoxyribonucleoprotein. For this reason it can provide a basis upon which to compare the performance of other staining techniques. The recently developed method for the staining of histones, based upon selective staining of basic proteins with the acid dye fast green at high pH's [8], affords a means for the estimation of the basic protein partner of DNA. Under certain conditions variations in the staining of histone with this technique can be made to occur which appear to reflect fundamental differences in the state of the cell during development [28] (see chromosomal proteins).

The elimination of most of the physical sources of error is a relatively simple task now that they have been recognized and evaluated. The two-wave-length method, as developed independently by Ornstein [145] and Patau [150], and the scanning technique of Deeley, *et al.* [56] have proved to be effective tools for the elimination of the most serious defect, the distributional error arising from the heterogeneous distribution of the chromophore within the cell. The numerous objections to the use of cytophotometry in determining the total amount of absorbing material, as set forth by Danielli [52], Glick, *et al.* [75], and Caspersson [39], are useful in that they draw attention to the various pitfalls awaiting the uncritical application of this approach. The importance of these errors as enumerated by these authors was often over-stated, however, and the flexibility of the technique in its ability to cope with these errors was underestimated. While the standards by which the performance of these microprocedures is judged may be below those desired in the classic types of analytical approach, their use is certainly justified by the valuable cytological information they have made available which at present could not have been obtained in any other way.

2. *Desoxyribonucleic Acid*

BIOLOGICAL PROPERTIES OF DESOXYRIBONUCLEIC ACID. Of the main components of the chromosome the principal candidate for genetic activity appears to be desoxyribonucleic acid. This substance has been strongly implicated ever since the development of the Feulgen reaction and the subsequent characterization of the chromosome and chromatin of the nucleus as containing DNA [67]. Its function in the transmission of hereditary characters is suggested by the similarity of its absorption spectrum and the mutagenic action spectrum in the ultraviolet [87]. Its physiological activity and hereditary role are stressed in the studies on the transforming principle: the substance which, taken from one strain of micro-organism, can induce hereditary traits characteristic of that strain to appear in a second strain. Since its discovery by Griffiths [78], and characterization as DNA by Avery, McLeod, and McCarty [13], the transformation of micro-organisms has branched into a whole new science of biochemical genetics. In transformation, the pure DNA fractions demonstrate phenomena simulating those of classical genetic studies, such as independent assortment [165, 200], linkage [92], all-or-none action [92, 165], alleles [198], crossing-over [199], even perhaps mutation (for review see Zamenhoff [217]). The recent work of Allfrey [10] and Gale and Folkes [72], implicating DNA in protein synthesis, provides a promising lead into the mechanism of genetic control over cellular processes. The molecular model of DNA postulated by Watson and Crick [212, 213], based upon considerations of the composition and structure of the molecule and supported by X-ray diffraction studies [69, 214], provides a basis for a scheme for the replication of DNA. The model consists of a double relationally coiled helix, each of the single strands consisting of a desoxyribophosphate backbone. The two helices are connected by hydrogen bonding through the bases, adenine to thymine and guanine to cytosine. This model offers an explanation for such phenomena as the one-to-one ratio of purines to pyrimidines as observed by Chargaff [40, 42], and the possible significance of the observations of the classical cytologists on chromosome coiling [73, 213].

In view of the remarkable stability of the gene, which can persist in the same form while undergoing all the vicissitudes of duplication and cellular metabolism with only the process of natural selection to protect it from change, it is not surprising that the rate of incorporation of new constituent atoms into DNA is among the lowest of any occurring in biological systems [34]. Since incorporation and DNA synthesis have been found to occur concurrently [93, 140, 202], this incorporation may be due not to metabolic turnover but to synthesis of new molecules. Paradoxically, however, DNA does display a surprising readiness to incorporate unphysiological bases into the molecule [218, 219]. The biological effects of such artificial nucleic acids have yet to be determined.

For a more extensive review of the biological activity of DNA the reader is referred to Hotchkiss [91].

DNA CONSTANCY. "It has become a textbook generalization that all cells of an organism have the same chromosomal constitution." This statement of Schrader and Leuchtenberger in 1949 summarizes the then prevailing attitude and was held by these authors to be inadequate in explaining the manifold changes of which the cells are capable during differentiation [178]. Nevertheless, the generalization has since been shown to apply in great extent to what might be regarded as the most important genetic constituent of the chromosome, the DNA. Analysis of this substance from the various tissues of a number of organisms has shown the amounts per cell (disregarding polyploidy and polyteny for the moment) and composition to be a species characteristic [112]. Chargaff and co-workers have shown the four purines and pyrimidines to occur in constant ratios within the tissues of any one species, regardless of tissue source [42]. Boivin and the Vendrelys [31] and Mirsky and Ris [135], using biochemical extraction procedures, have demonstrated that, with certain exceptions, the amount of DNA per cell is relatively constant within a species. The haploid germ cells contain half the amounts found in most somatic cells, while certain other tissues, such as mammalian liver, contain amounts above the normal complement.

Because many tissues contain mixtures of cells with various degrees of polyploidy,[2] the cytophotometric technique with its ability to measure the DNA content of individual cells is better adapted to the purpose of demonstrating DNA constancy per genome than are the extraction techniques, which are inadequate to treat the various groups of cells separately. The most extensive cytochemical work on the DNA content of the cells of various tissues is that of Swift [193]; the results obtained support the hypothesis of DNA constancy per diploid cell. His analysis of individual cells demonstrates that certain tissues contain cells with increased amounts of DNA in addition to those containing the basic diploid amount. These increases occur in a definite pattern, consisting of geometrically increasing integral multiples of the basic diploid amount, in the same manner that the number of chromosomes increases in a polyploid series. That such DNA variations are in fact due to polyploidy or polyteny is seen in the relationship between DNA content and the known degrees of polyploidy. The haploid germ cells invariably contain half the somatic diploid amounts of DNA [2, 179, 193, 202]. Corn endosperm, a polyploid tissue derived from a cell which results from fusion of a diploid egg and a haploid sperm nucleus, has a basic amount of DNA three times that of the haploid cell [194]. Clearly, then, the amount of DNA in a cell is proportional to the amount of chromosomal material contained within the cell. This is the basis of the concept of DNA constancy.

This concept has been the object of some controversy in the past, no doubt owing in part to the ambiguity of the term constancy. Probably no one upholds the view that DNA is completely the same, atom for atom, in every chromosome set. Variation in the numbers of chromosomes per cell over and above that due to polyploidy gives rise to aneuploid cells [83, 94], and a direct correlation has been shown between DNA content and chromosome number in such cells [208]. It is also possible that parts of individual chromosomes are lost or gained occasionally during division [105]. Whether such differences are due to errors in chromosome duplication or distribution or, as suggested by Huskins and others [96], to a natural process of cell differentiation cannot be answered at present. A number of reports describe variations in the amount of DNA among normal cells of different tissues [117, 121, 137, 148, 178]. This variation is purported to be outside the limits of experimental error. In several instances, however, these results have been refuted by other investigations using the same or similar material [9, 129, 195], and, in general, such results remain unconfirmed. Interestingly, several reports provide evidence that DNA variation occurs in such pathological phenomena as infertility [118, 119], and tumor growth [167]. These instances suggest the importance of a complete genetic balance for the *normal* operation of a cell, and appear to provide indirect evidence in favor of a high degree of DNA constancy among normal cells. It is significant that improvements of the technique of measurement have led to lower and lower standard deviations for the amounts of DNA per cell.

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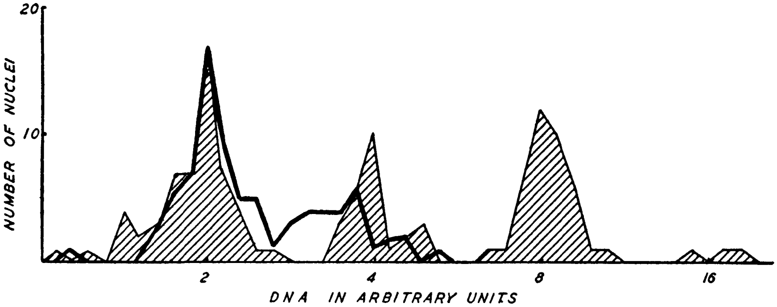
[2] The term polyploidy refers to the condition of having integral (other than two) multiples of the haploid chromosome number. Polyteny is a similar condition except that the homologous chromosomes are not separated. *Aneuploidy* and *hyper-* and *hypoploidy* describe the state of having other than integral multiples of the haploid number.

DNA DURING THE CELL CYCLE. The pattern of DNA increase in polyploid tissues reflects a peculiarity in the synthesis of this substance, that of self duplication. It was seen above that DNA increases geometrically, each polyploid cell containing twice the amount of DNA contained by cells of the class immediately preceding it in the series. In other words the amount of new DNA synthesized always equals the amount already present before synthesis occurs. This fact suggests that DNA, or the complex containing it, plays a role in its own synthesis. Although within any one cell type other properties may increase in a similar manner, such as protein [7, 28] and cell size [85, 98], it is the constancy of amount taken in conjunction with the geometric rate of increase in similar and different cell types which points to DNA as the initial site of replication.

The increased amounts of DNA per cell as shown by biochemical extraction procedures are not always to be interpreted as an indication of polyploidy. During tissue proliferation cells in the autosynthetic stage contain increased DNA, and if these cells make up a sufficiently large proportion of the population, their presence will be detected as an increased average DNA per cell [180, 154]. As in the case of polyploidy, cytophotometric analysis of individual cells can be used to resolve this increased DNA per cell into a pattern of DNA synthesis, relating this process to that of cell division. It is apparent that such increased amounts of DNA during proliferation are a necessary corollary to the concept of DNA constancy among resting cells.

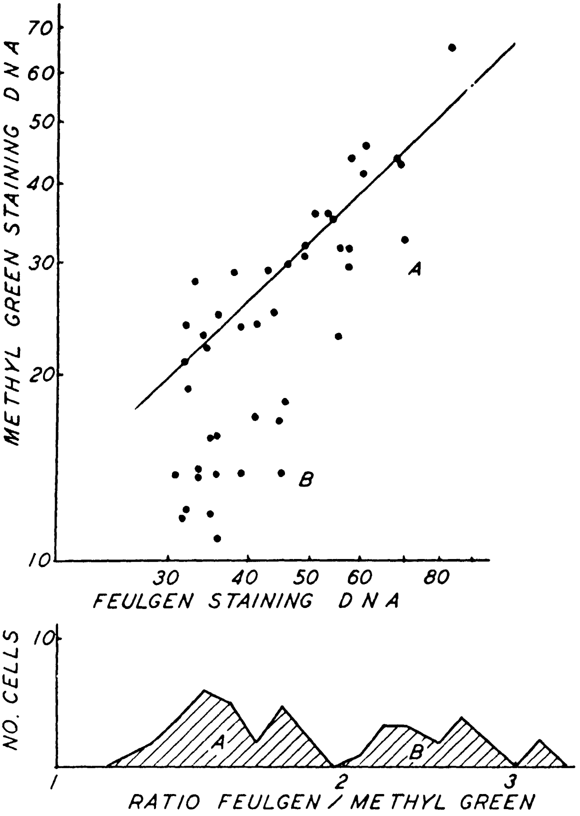
Instead of showing the normal single peaked distribution curve or polymodal curve with well separated peaks, the frequency distribution of amounts of DNA per cell drawn from rapidly proliferating tissues shows a curve containing the main peak at the diploid value, a subsidiary peak at twice this value, and a number of points intermediate between the two peaks (Text Fig. C). This behavior was first demonstrated by Swift [193, 194] using Feulgen stained material, and has since been shown by a variety of methods [16, 27, 70, 154, 202, 209, and others].



TEXT FIGURE C. Frequency distribution of the amounts of DNA contained in the individual cells of the embryonic (shaded portion) and adult (unshaded portion) mouse liver (Patau and Bloch, unpublished).

The cells with the doubled amount of DNA have recently completed synthesis of chromosomal material and are about to undergo mitosis. The intermediate values belong to cells in the midst of synthesis. The cells with the diploid amount have either not yet begun synthesis or are in the "heterosynthetic" interphase; that is, they are not taking part in proliferation. These are all interphase cells, indicating that DNA synthesis takes place during interphase.

Walker and Yates obtained more direct evidence for synthesis during interphase by watching a number of cells in tissue culture and measuring the amounts of DNA at varying intervals after mitosis by means of densitometric measurements of photographs taken with ultraviolet light [209, 210]. They found that DNA increases, approximately doubles, during an appreciable interval of the interphase period. By means of the two wave-length modification of the photometric technique Patau and Swift were able to measure the amounts of Feulgen-stained DNA in mitotic cells despite the extremely heterogeneous distribution of the chromophore in these cells [151]. Their results indicated that no DNA synthesis occurs during the period from late interphase through mitosis to next early interphase. Clearly, then, replication must take place during the interphase period. This was later confirmed by Deeley, *et al.,* using scanning techniques for determining the amount of DNA throughout the entire cell cycle [56].

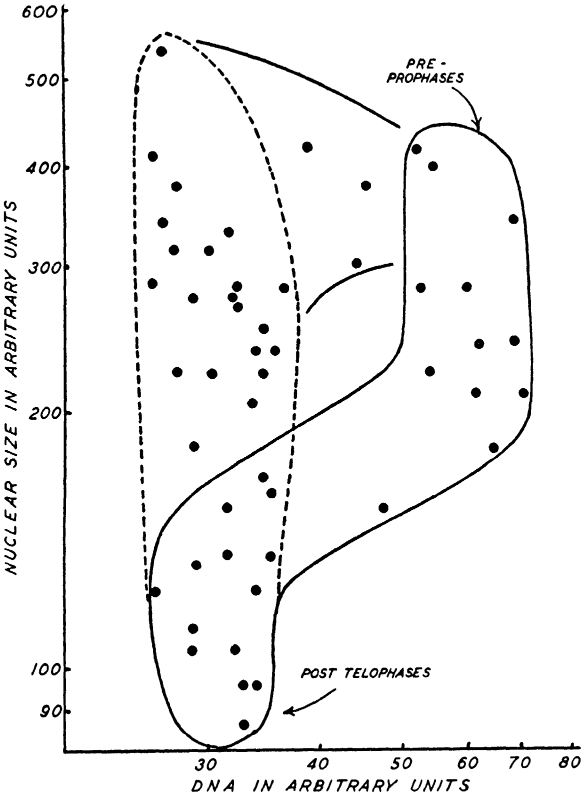


TEXT FIGURE D. Methyl green plotted against Feulgen staining of the liver nuclei of a young rat. Autosynthetic nuclei are those falling along the regression (A). The remaining nuclei are heterosynthetic (B). Both scales are plotted logarithmically and expressed in arbitrary units.

In many rapidly proliferating tissues, cells in the two types of interphase stages can be distinguished by consecutive staining and measurement of stain in the same cells, first with methyl green and then with the Feulgen reaction (Text Fig. D). During the autosynthetic interphase, the period of duplication of the chromosomal material, there is a partial increase in the amount of DNA, as shown by both stains, from the diploid to twice that amount. The heterosynthetic interphase is characterized by the diploid amount of DNA as indicated by Feulgen staining, but there is a distinctly decreased capacity to bind methyl green. That these are in fact heterosynthetic cells is seen from the identical staining characteristics of this group and the homologous non-proliferating cells of the adult animal [28]. The difference in the methyl green staining properties of these two interphase stages is probably not due to an inherent difference in the DNA itself but, as will be seen in the section on proteins, reflects a difference in the nature of the protein associated with DNA.

Correlation of amount of DNA with nuclear size gives a clearer indication of the pattern of synthesis during both interphase stages of the cell cycle. Alfert [2] and Patau [149] have shown that if DNA is plotted against nuclear volume in plant meristem an S-shaped curve is obtained. Following mitosis there is an increase in nuclear size, followed by a period of increase of both nuclear size and DNA content, then continued growth in size alone. This process is illustrated by the cells circumscribed by the S-shaped solid line in Text Figure E. In this case, liver of young rats was the material studied. Apart from the autosynthetic cells falling within the S-curve there are a number of large cells containing the diploid amount of DNA. These cells, shown within the broken lines, are in the heterosynthetic phase, as determined by both their morphological and staining characteristics. Their absence from the cell population in root meristem may account for the pure S-shape relationship between size and DNA content described by the abovementioned authors. Presumably a sample which included some nondividing cells from regions higher than the meristem might show a pattern similar to that of the liver. It is of interest that a few cells are pictured in Text Figure E which appear to be entering the autosynthetic period from the heterosynthetic, instead of from a previous mitosis. This may be the prevalent pathway during slow growth of a well-differentiated tissue.

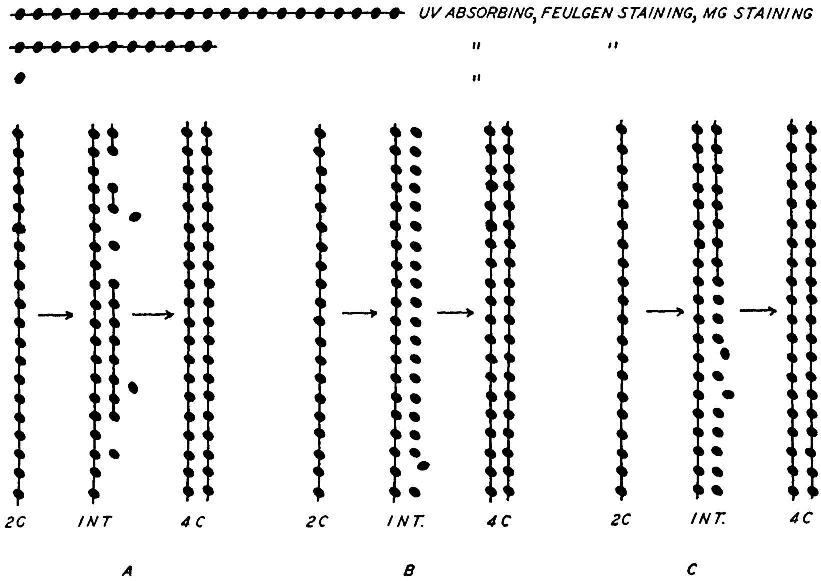
Additional evidence for DNA synthesis during the interphase is provided by studies on the incorporation of phosphorus into the DNA molecule. Howard and Pelc, using radioautographic techniques, showed that incorporation occurs during interphase [93]. Taylor and McMaster [202] and Moses [140], by coupling radioautography and cytophotometry were able to demonstrate that the period of incorporation coincides with that of DNA synthesis. Indirect evidence for synthesis during this phase is also provided by the relative rates of respiration, which generally reach a peak during the period immediately preceding mitosis [191, 222]; and by the susceptibility of mitosis to the action of respiratory inhibitors, which have their greatest effect if administered prior to mitosis [36]. The relationship between respiration and DNA synthesis is at present unknown. An energy coupling mechanism might not be required for the final steps of DNA synthesis, especially if the di- and triphosphorylated mononucleotides found in proliferating tissues are the immediate precursors of DNA [176, 177]. Such an energy-providing system, however, would most certainly be required for synthesis of the precursors.



TEXT FIGURE E. Nuclear size (volume expressed as area of the flattened nucleus) plotted against amounts of DNA. Nuclei within the solid S-shaped area are in the autosynthetic stage. Those bounded by the broken line are in the heterosynthetic stage. The few nuclei bounded by the upper solid lines have entered the autosynthetic from the heterosynthetic stage. Both scales are expressed in arbitrary units and are plotted logarithmically.

MECHANISM OF DNA SYNTHESIS. Cytophotometric techniques can also provide information on the gross mechanism of DNA synthesis from its nonpolymerized precursors. Walker and Yates were able to demonstrate the presence of a non-Feulgen-staining, ultraviolet-absorbing component of the nuclei of proliferating cells which is absent from cells that are no longer undergoing division [210]. This non-Feulgen-staining substance comprises an amount approximately equal to the Feulgen-staining DNA and behaves similarly during synthesis, doubling during interphase along with the DNA. Fractions of this material are lost when the cells are killed, fixed, and hydrolyzed; perhaps this fact accounts for.its failure to stain with the Feulgen reaction. Walker and Yates postulated that this substance comprises relatively unpolymerized DNA precursors, although they did not rule out the possibility of a nuclear RNA fraction behaving in this manner. Schmitz, *et al.*, have demonstrated by paper chromatography the presence of the various di- and tri-phosphorylated nucleotides in tumor tissues [177]. It seems likely that such substances might be directly along the line of DNA synthesis [128, 171] and may account for part of the non-Feulgen-stainable component of Walker and Yates.

DNA synthesis from such simple nucleotides may be visualized as occurring in a number of different ways (Text Fig. F). Perhaps the nucleotides fall into place, the internucleotide bond forming whenever two or more units fall into adjacent sites along a template, as illustrated in scheme A. Or possibly the bonds connect simultaneously only after a number of sites are occupied corresponding to a complete DNA molecule, or in the extreme case, to a complete genome (scheme B). Still another alternative would call for the consecutive linking of adjacent sub-units along the axis of the molecule (scheme C). While cytophotometric studies do not provide unequivocal evidence for any of these schemes, some of the available results do provide a basis for speculation and make certain of these schemes seem more likely than others. The binding of a number of dyes onto the DNA molecule is dependent upon the degree of polymerization of the DNA. This is true to a certain extent for all dyes, inasmuch as staining is contingent upon retention of DNA throughout the staining procedure, which in turn depends upon some degree of polymerization. It is especially true, however, of basic dyes such as methyl green, which have two binding groups per molecule. Kurnick and Mirsky found a threshold for the polymerization of DNA above which the methyl green-binding ability is unaffected. This threshold probably lies above 20 polynucleotide units [109]. There is also an unknown limit of polymerization, below which DNA no longer stains with the Feulgen reaction, if only because of the loss of these low polymers during hydrolysis and staining. This may account for the non-Feulgen stainability of Walker and Yates "precursors." The threshold of polymerization below which DNA no longer stains with the Feulgen reaction lies well below that for methyl green, as is demonstrated by the fact that enzymatic depolymerization of DNA affects methyl green to a greater extent than it does Feulgen staining, an increased ratio of Feulgen to methyl green indicating the degree of depolymerization of DNA [107].

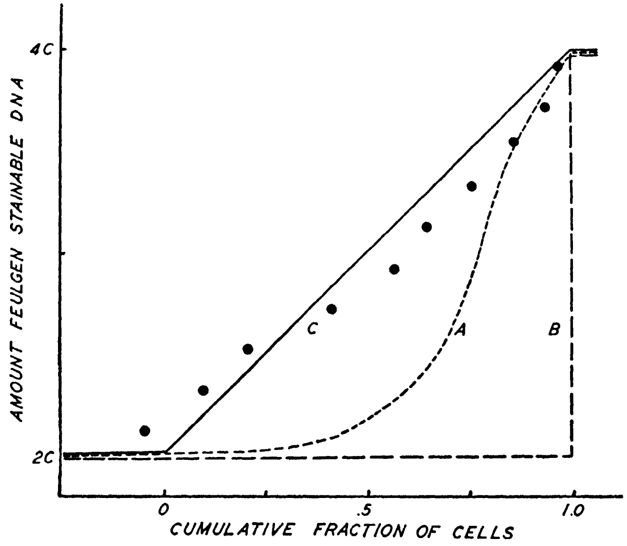


TEXT FIGURE F. Representation of several possible mechanisms for the linking of the internucleotide bonds during synthesis of DNA: (A) formation of bonds whenever two or more units fall into adjacent sites along a template; (B) simultaneous linking of the bonds after all the sites are occupied; (C) spatially consecutive bond formation.

Returning to the various postulated mechanisms for DNA synthesis it might be expected that if the first hypothesis is correct (that providing for the formation of the internucleotide bond whenever two or more nucleotides fall into adjacent sites), the initial stages of DNA synthesis would be characterized by an accumulation of low polymers. Some of these might be high enough to be retained during Feulgen staining, but low enough so that binding of methyl green is diminished. By comparing the Feulgen and methyl green uptake of the same individual cells during the various stages of DNA synthesis, Bloch and Godman found that the DNA, as indicated by both methods, increases proportionally during the autosynthetic interphase [28]. There is no evidence for changes in the amounts of low polymer DNA during the course of synthesis, as shown by changes in the ratio of Feulgen to methyl green staining.

These conclusions are corroborated by studies of the rate of DNA synthesis during the autosynthetic interphase. If scheme A is in operation and the orientation of the mononucleotides along the template occurs at an approximately linear rate, the rate of DNA synthesis (as shown by staining techniques whose intensity would depend upon the polymerization of these units) would (a) tend to be lowest in the initial stages of DNA synthesis when low polymers would be most prevalent; (b) tend to be highest in the intermediate stages when the addition of new units not only contributes to staining by itself but also results in the retention of previously unstainable low polymer chains; and (c) approach linearity in the final stages when addition of new units contributes proportionally to the staining of the polymers already present (see broken line, Text Fig. F). Therefore the rate of increase of stainable DNA during the various stages of the autosynthetic interphase can be used to test the validity of these hypotheses.

Walker has pointed out that the average time spent by a number of cells within any interval of DNA increment during synthesis should be proportional to the frequency with which cells are found within this interval [207]. If the number of cells falling within successive intervals are totaled, and if from the onset of synthesis time as a function of these sums is plotted against the amount of DNA, the accumulation of Feulgen-stained DNA is approximately linear with respect to time (Text Fig. G). Such results were first reported by Grundmann and Marquardt [79] and by Walker [208]. Although linearity is not exact, the deviations therefrom may be due to the difficulty in making accurate determinations of the frequency of cells within the increments bordering on the 2C and 4C peaks. They are at any rate in the opposite direction from what might be expected on the basis of scheme A (as shown by the broken line). A relationship between these types of results and the gross mechanism of DNA synthesis might seem rather tenuous on the basis of our present knowledge. It might safely be said, however, that they lend no support to scheme A and appear to favor scheme C. The fact that intermediates can be obtained at all would preclude an instantaneous synthesis of the complete DNA complement. Interestingly, a mechanism for DNA synthesis has been proposed by Watson and Crick



TEXT FIGURE G. Rate of DNA synthesis during the autosynthetic interphase, as shown by the frequency of occurrence of cells within the various DNA increments. Ordinate: amounts of Feulgen stained DNA. Abscissa: time, as a function of the cumulative fraction of cells falling within successive increments of DNA increase. Letters indicate hypothetical curves expected on the basis of the schemes described in Text Figure F.

[213] which might be expected to show such a linear relationship. That is, the reduplication of the single-stranded polynucleotide chains as they uncoil from the double helix. Such a scheme would be expected to result in the linearly sequential linkage of internucleotide bonds and a resultant paucity of low polymer polynucleotides.

These mechanisms were based upon the assumption that during synthesis, one DNA component remains intact while the second is built upon the first, the doubled genome consisting of an old and a new DNA fraction. Stevens, *et al.*, have measured the phosphorus uptake of cells during growth and found approximately twice as many atoms were incorporated per new cell as could be accounted for by synthesis of new DNA alone [192]. These results were interpreted as suggesting that during or prior to synthesis the DNA molecule is broken down, resulting in a replacement from the metabolic pool. These workers had estimated the number of new cells produced by the weight increase of the tissue, and by the use of colchicine to block cells which enter mitosis during the time that phosphate was administered. It will be shown below that colchicine treatment does not result in the static maintenance of the blocked cells, however, and it is likely that such treatment leads to an underestimate of the actual number of cells produced, thereby resulting in an overestimate of the amount of phosphorus incorporated per new cell. Fujisawa and Sibatani found evidence against phosphorus "turnover" during synthesis, using similar techniques [71]; and Barton, by incorporating isotopic phosphorus into DNA and then correlating isotopic dilution with growth, obtained evidence for retention of the phosphorus within the DNA molecule throughout DNA synthesis [71].

The most direct evidence for the maintenance of the intact polynucleotide during chromosome synthesis comes from the radio-autographic work of Mazia and Plaut, who demonstrated that if C^{14}-labeled thymidine is administered prior to cell division in *Crepis,* there is often found an unequal distribution of the labeled DNA among the two daughter cells after division [128]. These findings were interpreted as indicating that during chromosomal division the old DNA goes to one daughter chromosome and the new DNA to the other. Retention of label might not occur if there were a disruption of the polynucleotide during DNA duplication. Variation in the proportions of the old and new DNA distributed among the various cell pairs is purportedly due to the random assortment of the old and new chromosomes during division.

The mechanism of replication of the polynucleotide chain of DNA has been the subject of experiment and comment since the proposal of Watson and Crick [212] that reduplication might occur as the double helix unwinds, each strand acting as a template and resulting in two double helices. According to this scheme, each daughter chromosome would contain one strand of the original template polynucleotide for every strand of replicate.

A number of papers have since appeared which bear directly on the partition of old and new DNA among daughter cells and chromosomes. Some of the problems posed by a literal interpretation of the Watson-Crick scheme of DNA duplication are treated by Schwartz [226], Bloch [222], Butler [223], and especially by Delbrück and Stent [224]. Schwartz [226] and Bloch [222] take some liberties with the Watson-Crick view in order to reconcile the double helical structure of DNA with cytological and genetic observations. Whether such a reconciliation will come by way of modification of the model, or by reinterpretation of these observations is at present debatable. The recent experiments of Levinthal on bacteriophage [225] and Taylor, Woods, and Hughes [227] on plant material, dealing with the fate of parental and newly synthesized DNA through successive cycles of chromosome synthesis, give important evidence concerning the mechanism of replication of DNA. Both of these experiments appear to support the Watson-Crick scheme.

RELATIONSHIP BETWEEN DNA SYNTHESIS AND CELL DIVISION. Cytophotometry offers a unique means for demonstrating the relationships between cell division and the antecedent syntheses of chromosomal material. While these processes are certainly related, the capacity of a number of agents to block specific stages of the cell cycle suggests that a fair degree of autonomy exists between them.

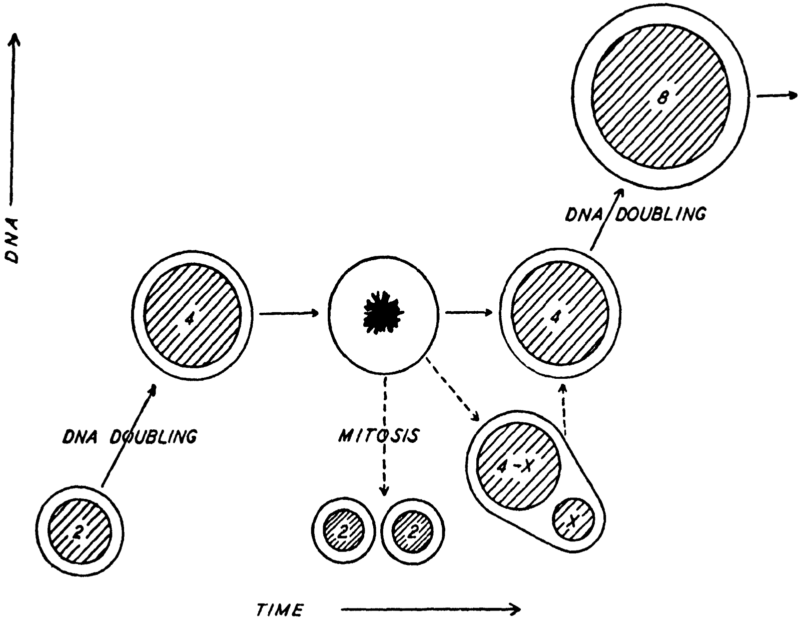
Woods [215] and Duncan and co-workers [62], studying the effects of purine and pyrimidine analogues on onion root meristem showed that 5-amino uracil inhibits DNA synthesis by competing with one or perhaps both of the pyrimidines. By cytophotometrically determining the relative frequencies with which the cells fall into the various DNA classes, and the mitotic indices, the following picture was obtained. Upon administration of the drug, DNA synthesis ceases. Mitosis, however, can proceed for a time because of the presence of a number of cells which had duplicated DNA prior to treatment. As these cells decrease in number, there is a concomitant decrease in the mitotic index. This suggests that the mitoses occurring during the intervening period result in the depletion of the cells with the doubled amount of DNA, and the mitotic block is primarily the result of the block in DNA synthesis. This would be expected on the basis of the assumption that DNA synthesis occurs during interphase. To go one step further, there was some indication, as shown by the relative lack of cells containing intermediate DNA values after treatment, that the drug does not inhibit DNA duplication once initiated but blocks the onset of duplication, perhaps taking effect on some reactions involved in the synthesis of DNA precursors. A similar effect was demonstrated by Swift, using X-irradiation and nitrogen mustard [196], and Duncan and Woods, using benzamidazole [62]. In these cases the "agents" blocked the onset of both mitosis and DNA synthesis, as shown by both the decreased mitotic indices and the frequencies of intermediate DNA values. That the latter block occurs at some time before the initiation of DNA duplication is also suggested in the studies of Duncan and Woods, by the fact that the most effective reversal of this inhibiting effect is brought about by administration of a mixture of thymine and folic acid. Probably both substances (certainly the latter) are involved in the synthesis of DNA precursors rather than in the assimilation of the precursors into the DNA molecule.

The investigations of Bodenstein and Kondritzer show that N-mustard, while inhibiting mitosis and DNA synthesis, allows growth, ribonucleic acid and protein synthesis, and differentiation to proceed at least for a time [30]. The implications of these studies are many. They certainly indicate that a fair degree of autonomy exists among what might be supposed the most regimented of all cellular processes.

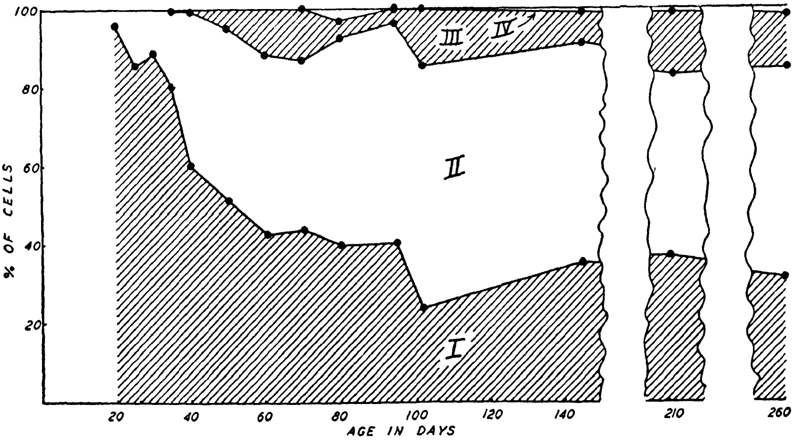
This lack of dependence of mitosis upon DNA synthesis appears to be carried to its extreme in the case of X-irradiated *Habrobracon* eggs, in which von Borstel has reported that division results in Feulgen negative cells [206]. Whether these cells are DNA deficient or the DNA has been so degraded as not to be demonstrable by Feulgen staining is debatable.

The reversed situation, the independence of DNA synthesis and mitosis, is seen in the well-known effect of colchicine on polyploidization [25] via spindle inhibition [97, 124]. Prolonged administration of colchicine to cultured cells results in a progressive increase in the percentages of polyploid cells as determined by cytophotometric analysis (Text Fig. H). DNA synthesis within the cell population appears to proceed at an undiminished rate during the initial stages of treatment. The cells revert from the metaphase to the interphase state with the higher DNA complement, and the continuous production of successively higher polyploids results until a general toxicity sets in [26].

The naturally occurring processes leading to polyploidy and especially polyteny serve further to demonstrate this independence. Text Figure I shows the increase in the proportions of the higher polyploid cells during development of the mouse liver. The fact that the "degree of polyploidy," or average amount of DNA per cell, is roughly proportional



TEXT FIGURE H. Schematic representation of the pathway of growth during colchicine inhibition of mitosis.



TEXT FIGURE I. Changes in the frequency of the various classes of polyploid cells, as indicated by amounts of DNA, during development of the mouse liver (Patau and Bloch, unpublished).

to both liver weight and weight of the mouse during this process demonstrates that the major though not total growth of the organ can be accounted for by polyploidy, and that the occurrence of this process does not decrease the growth potential of the organ [Patau and Bloch, unpublished]. This high degree of liver ploidy, incidentally, is lacking in the case of hereditary dwarfism in the mouse [115]. Sharp deviations from this curve occur during periods of moderate stress which may result in liver hypertrophy. Such an instance is pregnancy, as can be seen from the frequency distribution of DNA per cell in Text Figure C. The degree of polyploidy in this pregnant mouse is higher than any shown in Text Figure I. Such increases in polyploidy have also been found to occur during Salmonella infection, and during nonhepatic tumor growth in the rat [15].

3. The Chromosomal Proteins

If all somatic cells within an organism contain a similar genetic complement, as suggested by the apparent identity of the DNA fraction of these cells, the question of how differences arise among differentiated cells becomes the more pertinent. It might be supposed that during the course of development, changes are imposed upon DNA which can persist through duplication but which are too subtle to be detected with the chemical means at our disposal. Loss in biological activity of the transforming principle, for example, can be induced without resultant detectable changes in either the chemical or physical properties of the DNA molecule [220]. Fortunately, however, whereas such changes in the DNA itself may not be detected with the chemical methods now available, alterations occur in the desoxyribonucleoprotein complex which can be readily demonstrated by such methods. The universal occurrence of DNA in association with proteins suggests that these latter substances may play a role in regulating genetic expression. Although the significance of such changes is not certain, they appear to be correlated with changing physiological and perhaps also genetic function in the various stages of cell development. For this reason studies of the nucleoprotein complex or of the proteins themselves show considerable promise of contributing toward an understanding of the molecular events that lead from genetic control to synthetic activity in the differentiated cell.

At present our knowledge of the various chromosomal proteins is fragmentary and confused. They are perhaps best categorized on the basis of their chemical and physical properties, in spite of the fact that even these appear to be rather inconstant and ill-defined [155]. The two major categories consist of the basic proteins, or histones, and the acidic or nonhistone proteins. The histones are divisible into an acid-extractable and an acid non-extractable fraction [81, 133], which except for the presence of the sulfur-containing amino acids in the latter, appear to be similar in composition [81]. The acidic proteins consist of a tryptophane-rich protein [133], a residual protein [134], and a number of acid-soluble fractions such as those described by Wang, *et al.* [211] and Engebring and Laskowski [64]. The tryptophane-rich and the residual protein are considered identical substances by Mirsky [131]. The alkaline-soluble fraction may also include or be included within the residual protein [81]. Because of the reported properties and abundance in the cell, it is the author's opinion that the chromosomin of the Stedmans [187] may be a mixture of the acid-insoluble basic proteins and residual protein. Our detailed knowledge of these proteins is obviously rather meager. To what extent these fractions overlap, or could be considered "pure" from a standpoint of biological function, is doubtful. For these reasons such a classification as presented here must be regarded as tentative.

HISTONE. This basic protein has long been known to occur in the nuclei of higher organisms in association with DNA, as shown by extraction of the desoxyribonucleohistone complex [106, 130]. The reality of this association within the nucleus is evident from the closely similar distributions of both DNA and histone in the fixed and stained cell (Fig. 56). It is probable that these or similar proteins occur in all nuclei of higher organisms. The sperms or male gametes of a number of species, including fish [106], fowl [51], plants [48], and invertebrates [95], contain protamine, or proteins seemingly intermediate between the histones and protamines. These latter substances are extremely basic and have chemical properties sufficiently different from the histones to have earned them a special classification. In view of the great variability in the composition of the histones, however, it appears that protamine might simply be regarded as an extreme form of histone. Substances very much like the classical protamines are found in tissues other than sperm, although their nuclear origin is not assured [172]; and histones are found in the sperm of some species [106]. Thus there is no clear evidence that the two proteins are not homologous substances.

The chemical and physical properties of these two proteins similarly overlap. The sole or predominating basic amino acid in the protamines is arginine [106]. Histones contain all three basic amino acids, lysine predominating in some fractions [50] and arginine in others. The protamines are generally of low molecular weight, the larger molecules approaching 12,000 [65]. The histones, while on the average larger than the protamines, contain fractions having a molecular weight below 10,000 [1].

Histone displays the type of behavior which might be associated with genetic activity—although to a lesser extent than does DNA—and has been regarded by the Stedmans [188], Stern [190], and others [52, 90] as a likely candidate for the role of gene modifier during differential development of the cell. The most precise formulation of this view is that of Danielli [52], who suggests that histone can serve to restrict the synthetic functions of nucleic acids by combining with the phosphate groups. Such a genetic inhibitory role for the histones carries certain implications. The histone might be expected to vary in composition and amount from cell type to cell type, perhaps in such a manner that the amount or basicity of the histone is inversely correlated with the variety of intracellular synthetic processes. There should be a constancy of amounts of histone among cells of the same type, and among cells with approximately the same number of enzyme-catalyzed reactions. The amounts of histone in a species of organism should be roughly proportional to the number of cellular reactions which are not common to all cells within the organism. (Implied in these statements is the concept of cell specialization during differentiation as an increasing number of nonoperational genes.)

The relatively little that is known of the histones seems to support this general idea. Histone does not vary in amount per cell as much as do nonchromosomal proteins; neither is this fraction entirely constant. Like DNA, histone consists of a number of fractions differing in composition. Unlike DNA, however, such histone differences occur between cells of the various tissues from the same organism [35, 45, 50, 51, 63, 76, 77, 80, 82, 104, 123, 138, 188]. Nevertheless, a number of studies, both biochemical and cytochemical, stress a relative degree of histone constancy, at least within cells of the same type [8, 27, 135, 203]. Perhaps, as suggested by the Stedmans [188], the histone complement and therefore the desoxyribonucleohistone complex is characteristic of a cell type, much as DNA is a species characteristic.

With regard to metabolic activity, as expressed by turnover or incorporation of its constituent components, histone appears to be intermediate between DNA and the nonchromosomal proteins [49]. Incorporation of amino acids occurs in the absence of division [12] and hence cannot be attributed solely to duplication of chromosomal materials, but possibly reflects a "retooling" process necessary for a change in cell function. To what extent this incorporation involves a change in the composition of the histone fraction or synthesis of additional histone is not known at present.

Several investigations appear to show a more direct relationship between changes in the histone complement and the process of differentiation. Horn and Anderson have demonstrated a proportional increase in both DNA and histone during early embryonic development of the chick, followed by an increase in the ratio of histone to DNA during later development as extensive differentiation occurs [90]. Tumor growth is usually accompanied by a process of "dedifferentiation." Cruft, *et al.* [47] have reported a decrease in the amounts of histone in tumor tissue as compared with tissues from which the tumor was derived. Similar results have been obtained by others [153]. The increased basicity of the nuclear basic proteins of such highly specialized and synthetically inactive cells as nucleated erythrocytes and sperm is in accord with the hypothesis that histone acts by inhibiting the means by which the gene directs the synthetic processes of the cell [52].

Such a concept of histone function as a gene regulator has an interesting corollary. Proceeding down the evolutionary scale from the most highly organized to the simplest of organisms, it would appear that the need for regulation of genetic activity would diminish with decreasing complexity. On the one hand, differentiation among cells of the higher organisms is two dimensional, occurring with time as ontological development proceeds, and between the cells of the various tissues at any one time during development. In a colony of unicellular organisms, on the other hand, differentiation of a sort occurs in one dimension only, that of time. In this case determination of genetic activity is for the most part merely a function of the developmental stages which are common to all the cells of the colony, taking effect, for example, during the different phases of growth, or during enzymatic adaptation, in response to changing environmental conditions. The success of such a colony does not depend upon a coordinated divergent evolution of a number of subsystems, and cell differentiation is in this sense nonexistent.

There are no data available at present which would permit any conclusions regarding a correlation between the complexity of an organism and abundance of cellular histone. This substance is present, as shown by staining techniques, in such lower multicellular organisms as the nematode and basidiomycetes, but is not apparent in yeast [Bloch, unpub.]. Chargaff has reported a nonhistone association with DNA in avian tubercle bacilli [41]. Evidence for histone has been obtained, however, from pneumococcus by extraction [133], and from the single-celled protozoan *Tetrahymena* by staining techniques [5]. Obviously no generalization may be made of the presence or absence of histone in single cell organisms. If the purpose of histone is to determine gene action, the above fact would serve to increase the scope of such a regulatory role to include processes other than cell differentiation in the sense applied to development in the higher organisms, and might include modulation, and whatever other changes occur in the cell which depend upon genic mediation.

Histone changes do in fact occur in the cells of higher organisms, and these changes seemingly relate to genetic changes in the absence of real differentiation. Such relations will be seen in the section on proteins during the cell cycle.

NONHISTONE, OR RESIDUAL PROTEIN. Considerably less is known of the nonhistone component of the chromosome. Most of this fraction is probably accounted for by the residual protein [131, 132] which derived its name from the fact that this substance, along with RNA, makes up the bulk of the remaining chromosomal material after removal of both DNA and histone from isolated chromosomes. The residual protein appears to function in both a structural (see next section) and a metabolic capacity in nondividing cells. The latter is especially impressive. Incorporation of labeled molecules into this fraction is much higher than that into either DNA or histone, approaching that of the cytoplasmic proteins [49, 86]. Residual protein varies in amount from cell to cell, its physiological activity reflected by the fact that the amount present parallels the amount of cytoplasm and cytoplasmic activity. Thus residual protein accounts for approximately 39 per cent of the bulk of the liver chromosome, but only 4 per cent of the carp erythrocyte chromosome [135]. In this respect the behavior of the residual protein appears to be directly opposite that of the histone, which varies inversely with synthetic activity. The amounts of nuclear RNA and residual protein also run roughly parallel, as is seen by comparison of the data of Mauritzen, *et al.* [125] and of Mirsky and Ris [135] on the occurrence of the respective substances in comparable tissues. Their concurrence in the residual chromosome and mutual identification with metabolic activity suggest a possible association of the two substances within the chromosome.

Mirsky and co-workers [133, 136], and Mazia [126] have found evidence for a chemical association of residual protein (or in the latter reference, a nonhistone protein) with DNA. In spite of the fact that the residual protein is less basic than the histone [134], there appears to be a closer chemical association between the DNA and residual protein than occurs in the corresponding DNA-histone complex. This suggests that the nonhistone protein is bound to the DNA by means other than, or perhaps in addition to the saltlike linkages which are presumed to characterize the DNA histone complex [28, 133].

It appears likely that the structural element present in the residual chromosome, while included within the residual protein fraction, represents a separate entity from the metabolically active component. If the giant polytene chromosomes found in some species of Diptera are a valid representation of the ordinary somatic chromosomes with respect to the various components, the interband regions would bespeak such structural function and metabolic inactivity. Whether this dual role for the residual protein reflects our present inability to distinguish chemically between two different protein fractions remains to be ascertained.

CHEMICAL STRUCTURE OF THE CHROMOSOME. A clear resolution of the chromosomal components is made difficult by our inability to define these parts rigidly from either a chemical or a physiological standpoint, especially with regard to the protein fraction. A number of investigations have been carried out with the purpose of determining wherein lie the structural elements of the chromosome. Most of these studies were carried out on fixed or otherwise altered material. The components responsible for the continuity of the fixed or nonliving chromosome may differ from those holding the living structure together. Nevertheless the suggestions made by such studies cannot be discounted in the absence of data obtained by other means.

Mirsky and Ris were able to demonstrate that the basic structure of chromosomes remains intact after isolation from tissues. The chromosomes are recognizable as such, retaining some of the distinguishing characteristics by which the original chromosomes could be identified [134]. Lamb contested their view, considering the structures produced by such isolation techniques to represent relatively undefined nuclear fragments rather than discrete chromosomes released upon rupture of the nuclear membrane [111]. Denues however provided a convincing argument in favor of their chromosomal nature, based primarily upon an analysis of the morphological similarities between these structures and the chromosomes of the mitotic cell [58, 59].

When such isolated chromosomes are subjected to acid extraction resulting in the removal of histone, they retain their morphological identity. If the residual protein is then removed by enzymatic digestion leaving only the nucleic acid, the structure disappears. Enzymatic removal of DNA from the histoneless structure, or salt extraction of the DNA-histone complex from the complete chromosome, leaves a small thread of residual protein ("residual chromosome") bearing little resemblance to the original chromosome. These observations prompted the hypothesis that the DNA-residual protein complex is necessary for chromosome structure [136]. However, a similar role for the desoxyribonucleohistone complex cannot be ruled out. This substance has not been dissociated from the residual protein in its native state. The salt extraction used by Mirsky results in a dissociation and reassociation of the desoxyribonucleohistone [23].

Mazia and co-workers concluded that nucleohistone is sufficient to maintain chromosome structure [127]. They based their belief on the observation that pepsin removed the bulk of the chromosomal protein, presumably the residual protein, since histone—by virtue of its chemical composition and association with DNA—seemed relatively resistant to pepsin digestion. It is likely, however, that some histone loss occurred, because of both the acid extraction and the action of pepsin [51], and that the brief pepsin digestion used in these experiments did not result in the removal of all the residual protein [28]. Whether the structure of the chromosome can be maintained by the DNA-histone complex alone, undegraded by the action of strong salt solutions, is an open question.

There is no conclusive evidence that any single component of the chromosome acts as a string along which the other elements are oriented. Bernstein and Mazia showed that upon extraction of chromosomes with water at low ionic strengths, after removal of divalent ions, the desoxyribonucleoprotein is removed and can be recovered in the form of discrete particles about 4,000 A long and 200 A in diameter. These particles consist of DNA associated with histone and nonhistone protein in about the same proportions as they exist in the cell [22, 23, 126]. Whether these particles make up the chromosome *in toto,* whose structure would then depend upon the linear aggregation of these units, or whether their removal depends upon their dissociation from a "backbone," is not certain. Steffenson's observation that calcium deficiencies result in chromosome breakage in the intact organism [189] suggests that the ions which hold the desoxyribonucleoprotein particles together also hold the living chromosome together, and that these units may represent the total chromosomal material [126]. On the basis of such experiments, it would appear that the responsibility for chromosome structure can be relegated to no one of the components of the chromosome. Perhaps, as suggested by Mazia, such structure can be maintained by any of the components, each playing the dual role of structure and metabolic function [127], or perhaps structure in the living cell is dependent upon all of several components.

The particulate nature of the desoxyribonucleoprotein extracted from the chromosomes is suggestive of a similar type of organization in the living chromosome. The significance of such particles is questionable, however. It is unlikely that they could represent "ultimate genetic units" by all standards of definition. Benzer has found evidence of recombination occurring in bacteriophage between units a dozen nucleotides distant [20], a spacing which would occupy a much smaller volume than the particles of Bernstein and Mazia.

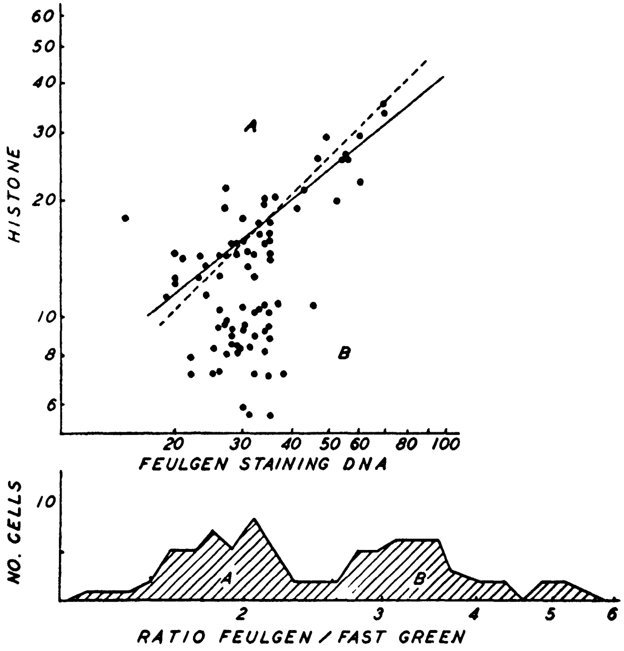
At the molecular level there is some evidence of the manner in which DNA and the various protein components are combined. Feughelman, *et al.,* draw the following picture of the desoxyribonucleoprotamine complex on the basis of X-ray diffraction studies and knowledge of the composition of this material [66]. The protamine is fully extended along regions where uninterrupted polyarginine chains occur, these chains forming a third helix relationally coiled around the double polynucleotide helices of Watson and Crick. The intervals between the amino acid residues is about one half that between the successive nucleotide units. Adjacent arginine residues point in opposite directions, the basic groups combining alternately with the phosphate groups of the two polynucleotide helices, thereby forming a bond between them. There appears to be almost one arginine residue for every nucleotide phosphorus [65], a "stoichiometric" relationship between the two groups. Since, however, the protamine consists of only two-thirds arginine, Feughelman, *et al.,* suggest that consecutive nonbasic amino acid residues are looped in an otherwise extended molecule in such a manner that they do not lie parallel to the polynucleotide chain, and therefore do not interrupt the spatial sequence of arginine phosphate bonds.

Riley and Arndt found that the desoxyribonucleohistone complex occurs in the form of a simple chemical addition product, histone retaining its globular nature upon interacting with the polynucleotide. The artificially prepared and naturally occurring complexes are similar in this respect [168, 169]. The difference between the fibrous and globular nature of the protamine and histone, respectively, may be one of degree rather than of kind. The histone molecule has a much greater proportion of nonbasic residues than the protamine, and if both types of proteins were to combine with DNA in the manner suggested by Feughelman *et al.*, the abundance of the nonbasic loops may enable the histone to assume a predominantly globular form.

There is no direct evidence of the physical configuration of the residual protein. This substance is relatively difficult to extract, and quite insoluble under conditions which do not degrade proteins, and has for these reasons been assumed to have a fibrous configuration. As will be shown below, this protein is strongly bound to the DNA molecule, being a very effective inhibitor of basic staining of DNA in spite of its relative acidity as compared with the histones.

NUCLEOPROTEIN DURING THE CELL CYCLE. Photometric measurements on cells during the various stages of the cell cycle, successively stained with a variety of techniques, show changes which indicate alterations in the state of the desoxyribonucleoprotein complex during these stages. By consecutive staining and measurement of the same cells with both the Feulgen procedure for DNA and Alfert and Geschwind's alkaline fast green method for histone, determinations of both substances can be made during identical stages of chromosome synthesis [27]. By this procedure, it was found that in the cells of a rapidly proliferating tissue both DNA and histone undergo simultaneous doubling prior to mitosis [7, 27]. Whether this fact reflects a mutual dependence of the syntheses of these substances or is merely the result of a parallel time sequence is not known.

If a similar procedure using methyl green followed by Feulgen staining is applied to cells of a young rat liver (both stains specific for DNA), the cells fall into two groups on the basis of their staining characteristics. These two groups comprise the aforementioned auto- and heterosynthetic interphases respectively. As previously described in the section dealing with DNA during the cell cycle (Text Fig. D), during the autosynthetic interphase there is a parallel increase in both methyl green and Feulgen staining as the DNA doubles in amount in preparation for division. The heterosynthetic interphase, however, is characterized by



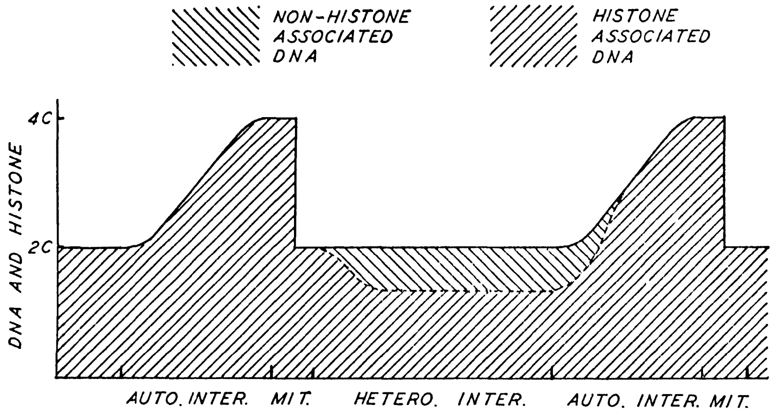
TEXT FIGURE J. Fast green staining of histone after methanol fixation, plotted against Feulgen staining of DNA, in the liver of a young rat. Autosynthetic nuclei fall along the regression, the remaining nuclei are in the heterosynthetic stage. Both scales are plotted logarithmically and are expressed in arbitrary units.

the 2C amount of DNA, as indicated by the Feulgen measurements, and by a decreased capacity for methyl green binding [28]. This inhibition of staining is not due to a decreased degree of polymerization of DNA during this stage. Staining is enhanced by acid extraction of histone, by pepsin hydrolysis, by formalin fixation, or by acetylation. The latter two treatments, according to Olcott and Fraenkel-Conrat [144], result in the blocking of protein-basic groups. All these treatments act by eliminating protein as a source of competitive inhibition of nucleic-acid staining.

Differences between the staining characteristics of the auto- and heterosynthetic interphase cells appear to reflect differences in the types of protein associated with the DNA during these stages. Removal of histone shows that this substance inhibits methyl green staining during all stages of the cycle. However, an additional inhibitor remains in the heterosynthetic cells, the effects of which can be circumvented by prolonged pepsin hydrolysis or acetylation [28]. This nonhistone inhibitor of methyl green staining appears to share a number of properties in common with the residual protein. It is probably less basic than the histones, as shown by its inability to bind fast green under conditions for histone staining. In spite of this fact, it binds more tenaciously than the histone to the DNA. It remains in the nuclei after treatments which are supposed to extract all protein but the residual protein [28]. The inhibitor is absent or occurs in imperceptible amounts in nucleated erythrocytes, cells known to contain small amounts of residual protein [135]. A similar capacity of residual protein for inhibiting basic staining of chromosomes isolated from nondividing (corresponding to heterosynthetic) cells was also noted by Mirsky and Ris [136].

A modification of the alkaline fast-green technique of Alfert and Geschwind for staining histone also provides evidence of alteration of the histone or the desoxyribonucleohistone complex during the two interphase stages of the cell cycle. If tissues are fixed with methanol instead of formalin, part of the histone fraction of the heterosynthetic cells is either lost or rendered unstainable. Consecutive staining of young rat liver with Feulgen and fast green after such fixation shows that the cells now fall into the two groups (Text Fig. J). Among the autosynthetic cells there is the parallel increase in DNA and histone from the 2C to the 4C amounts. The heterosynthetic cells are characterized by the 2C amount of DNA, but also by a decreased capacity to bind fast green by the histone.

The marked similarity between this behavior and that encountered in the methyl green and Feulgen staining suggests that the decreased staining of both histone and DNA during the heterosynthetic interphase stage are separate manifestations of related events. Consecutive staining of the same preparations with methyl green, followed by Feulgen and then fast green, demonstrates that the cells showing the decreased capacity to take up cationic methyl green and anionic fast green are identical. These stains are similar in that both bind by saltlike linkages to their respective substrates. However, it is not altogether certain that the staining inhibition in the case of fast green can similarly be attributed to a competitive interference. Presumably such interference could come from nucleic acid or other proteins. In this case, however, nucleic acids are ruled out, since histone staining depends upon prior extraction of these substances [8]. Histone staining is subject to interference from other proteins, at least *in vitro* [27], so that interference from this source remains a possible explanation. But the possibility also exists that histone is more easily lost from portions of the chromosomes in which DNA has become associated with the nonhistone protein, its loss depending upon the type of fixation.



TEXT FIGURE K. Changes in the desoxyribonucleoprotein complex during the various stages of the cell cycle, based upon changes in staining behavior.

The behavior of the desoxyribonucleoprotein complex during the course of the cell cycle, as interpreted from the results of such staining experiments, can be summarized as follows (Text Fig. K): during rapid proliferation, whether occurring over the whole of a tissue (plant meristem) or within a fraction of the cells of a tissue (young rat liver), most of the cells involved proceed directly from mitosis to the autosynthetic interphase with no—or at most a very transient—intervening heterosynthetic period. This process describes the S-shaped curve seen in Text Figure F when DNA is plotted against nuclear size. During such an autosynthetic period the DNA appears to be completely associated with histone throughout replication. It is not unlikely that a structural "residual protein" is present in the chromosome during this phase of growth. However, there is no evidence that this substance is bound to the DNA in such a manner as to interfere with staining. If after mitosis the cell enters into the heterosynthetic interphase stage, during which it presumably functions in the specialized manner commensurate with its state of differentiation, a portion of the DNA appears to become associated with a nonhistone protein, possibly the metabolically active fraction of the residual protein, which inhibits the basic staining of the DNA. Meanwhile, part of the histone fraction is either rendered easily extractable or becomes associated with other proteins in such a manner that its staining properties are also affected.

During slower growth, as the cells enter again into the autosynthetic interphase, this time from the heterosynthetic stage, the nonhistone protein becomes dissociated from the DNA, and the complex with histone again becomes the predominant form. By the time the chromosomal complement is fully duplicated, the 4C stage, the DNA histone complex exists in its "pure" form, as shown by the maximum staining of both the DNA after histone removal and the histone itself after DNA removal.

When this stage is attained, although the nonhistone protein may be dissociated from the DNA, it might still remain in the vicinity of the chromosomes. For example, Jacobson and Webb [99] and Kaufmann and co-workers [102], using staining techniques, found evidence of a charge of ribonucleoprotein in the chromosomes during mitosis. Lack of RNA basophilia during earlier stages cannot be construed to mean an absence of this substance. As with DNA, the basic staining of RNA is subject to competitive interference. Therefore changes in basophilia must be interpreted with caution [84, 163]. In spite of the fact that the ribonucleic acids appear to stain during mitosis slightly later than the apparent dissociation of the DNA-nonhistone complex, it is tempting to speculate about the possibility that during the earlier stages the protein associated with RNA may have acted as the DNA- and histone-staining inhibitor. If so, the elimination or sloughing of a part of this substance from the chromosome during anaphase is further indication that by this stage it has become extrachromosomal.

Recently Alfert has demonstrated that the staining properties of DNA and histone undergo changes during a process of pycnotic degeneration in ovarian follicles which simulate those occurring as the cell proceeds from the heterosynthetic to the autosynthetic and mitotic stages. As pycnosis progresses, the ratios of both methyl green and fast green to

Feulgen staining increase [6].[3] That pycnosis might resemble mitosis in this regard, and perhaps also the period during chromosome replication, is not surprising in view of the similarities of the two processes with respect to lack of synthetic activity and the density of the chromosomal material, as pointed out by Leuchtenberger [114] and Pollister [155].

Variability of the chromosomal nucleoprotein complex during the different stages of the cell cycle is partially substantiated by biochemical studies of a preliminary nature. Rotherham, *et al.* [173] found that the desoxyribonucleoproteins of rat liver could be separated into two fractions, on the basis of their differential solubilities. The relative prevalence of the two depended upon the proliferative activity of the tissue.

4. Biological Role of the Nucleoprotein Complex

The varied nature of the desoxyribonucleoprotein complex during the cell cycle poses the question of whether these observed alterations reflect changes in physiological activity at the level of the gene. During mitosis, and perhaps to a lesser degree during chromosome replication, while the DNA-histone complex prodominates and part of the nonhistone protein is seemingly dissociated from the DNA, there is a retardation or cessation of many of the physiological processes associated with specialized cell function. It would appear then, that DNA is associated with the relatively inert histone fraction during periods of genetic inactivity; and with a nonhistone protein, possibly the metabolically active fraction of the residual protein, during periods of genetic activity. If the types of protein associated with DNA are related to genetic function, the question arises whether the changes in association as indicated by the changes in staining are relegated to a specific fraction of the genes within any given cell type, perhaps the "operational" ones, or whether these changes occur "diffusely" over the entire genome. Direct comparison of the staining behavior of specific regions of the chromosome provides no definite answer at present. However, consideration of a number of investigations dealing with changes in chromosome chemistry and morphology during the various aspects of cell life suggests a partial answer to this question.

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[3] This may not be generally true of pycnosis. Leuchtenberger found that methyl green staining decreases in relation to Feulgen staining during pycnosis occurring in degenerating transplanted tissues [114]. The differences in these results might be explained by postulating that in the former case the predominating feature is a dissociation of a DNA-nonhistone complex, while in the present case a depolymerization of DNA occurs; the effect of depolymerization on decreasing the binding of methyl green by the DNA overrides any opposing tendency by changes in the DNA-protein association.

Beerman [19] and Pavan and Breuer [152] have shown that certain bands of polytene dipteran chromosomes undergo morphological changes during development which are characteristic of both developmental stage and tissue type. Such studies seem to implicate individual loci on the chromosome with specific physiological function, a conclusion which, of course, accords with many of the present concepts of gene action. The changes observed involve an expansion of definite bands and a resulting diffusion of the chromatin material within the band. The changes suggest an association of an additional substance with the gene during this stage. These regions appear to be similar to the "puffs" pictured by Caspersson as playing an important role in protein synthesis [38]. Additional evidence for a role of specific regions of the chromosome during synthetic cellular processes is seen in the lampbrush chromosomes found in the oocytes of various vertebrates. Numerous regions of these chromosomes, marked by outcroppings in the chromonemata in the form of loops along an apparent chromosomal axis, appear to be involved in the extensive synthesis which takes place in the developing oocyte. These loops consist primarily of a Feulgen-negative material in which presumably an extended Feulgen-positive strand is imbedded. The loops are perhaps the diploid counterpart of the Balbiani rings and puffs of the giant polytene chromosomes. For a discussion of the structure and the biological significance of these chromosomes the reader is referred to Ris [170] and Alfert [4].

Whether the apparent accumulation of material in these sites of the chromosome during active synthesis is in any way related to the inhibition of the staining of DNA and histone with cationic and anionic dyes in the heterosynthetic nucleus is an open question.

Rudkin [174] has recently reported an increase in protein with no change in nucleic acid content during development of the puffed regions in the polytene chromosomes. Caspersson [37] has characterized these regions as rich in proteins of the histone or "diamino acid" type, on the basis of slight shifts in the protein absorption peaks in the ultraviolet allegedly typical of basic proteins when combined with nucleic acids. The staining inhibition during the heterosynthetic stage, however, was seen above to depend upon the presence of a relatively nonbasic protein. It is likely that Caspersson's characterization is erroneous. Mirsky and Pollister [132] observed no such shift on comparing the absorption spectra of nucleohistone and a nucleic acid-albumin complex. Caspersson has similarly characterized the nucleolus as rich in basic proteins; however, these organelles have in fact been shown to be relatively devoid of these substances by Vincent [205], using isolation procedures, and by Pollister and Ris [164] and Alfert and Geschwind [8], using staining techniques.

In the genetic mediation of cellular processes there must be some point at which a chromosomal constituent becomes a chromosomal product, using the term "constituent" in the broadest sense to include substances assimilated by the chromosome or assimilated elsewhere and given specificity by temporary association with the chromosome. The identity of such immediate products of chromosomal activity in the differentiated nondividing cell and their relationship to more remote cytoplasmic processes are unknown at present. Consideration of a number of sequences at the morphological and biochemical levels are useful in providing a discontinuous though not entirely obscure picture of the chain of events connecting genetic with cytoplasmic activity.

According to Caspersson's scheme for protein synthesis, based upon ultraviolet absorption studies of cell constituents during cell development [38], synthesis of nucleolar ribonucleoprotein through association with the organizing regions of the chromosome, and of nonnucleolar chromosomal ribonucleoprotein, precede the synthesis of nuclear proteins. These latter substances diffuse toward the nuclear membrane, and at the perinuclear region of the cytoplasm, cytoplasmic ribonucleoproteins are synthesized. Cytoplasmic protein synthesis follows. Similar observations have been described by Lagersted [110], who used staining techniques to follow qualitatively the synthesis of nuclear and cytoplasmic RNA and cytoplasmic proteins. Such a sequence was also described by Taylor [201] on the basis of radioautographic studies. Labeled phosphate administered to *Drosophila* larvae was found to be taken up first in the nucleolus and certain regions of the chromosome, the activity of the latter being highest. Incorporation into the cytoplasm followed. Most of the incorporation occurred in the RNA fraction. These findings appear to support Caspersson's scheme in principle if not in exact detail.

Metabolic studies of a more conventional type suggest a sequence which is quite compatible with the foregoing scheme. Evidence for the direct involvement of DNA in protein synthesis was obtained by Allfrey [10], who demonstrated an incorporation of amino acids by isolated nuclei which depends upon the presence of DNA, but not RNA. The nature of the protein formed was not shown. Gale and Folkes found that enzyme synthesis could be induced in disrupted bacterial cells with the nature of the enzyme depending indirectly, at least, upon the type of nucleic acid present [72]. They found an RNA fraction to be necessary for catalase synthesis, while synthesis of β-galactosidase (an adaptive enzyme in the organism studied) depended upon the presence of DNA also. In the latter instance synthesis of RNA occurred and the authors postulated that this substance at least intermediates in the synthesis of the enzyme, the DNA perhaps inducing synthesis of the RNA. This fact appears especially significant, in that it stresses the need for genetic mediation at the highest level, that of the chromosome, where qualitative changes occur in the enzymatic constitution of the cell.

The immediate products of DNA activity are unknown. Whether such products fall into the residual protein fraction remains to be determined. The high rate of amino acid incorporation into this chromosomal fraction [49, 86] might point to either a rapid turnover of a "static" protein or continuous synthesis of new protein. It seems likely that such a protein (or fraction thereof) in association with the RNA of the residual chromosome might act as an intermediary in the synthesis of cytoplasmic ribonucleoproteins.

There are a number of examples demonstrating the importance of the nucleus in the synthesis of cytoplasmic RNA and protein [33]. Brachet and Linet have demonstrated a loss in basophilia in anucleate halves of *Amoeba proteus* which does not occur in the nucleate halves [32, 120]. The incorporation of labeled molecules into nuclear RNA is higher than that into the cytoplasmic counterpart and precedes it with respect to time [21, 101]. Shemin and co-workers found that heme synthesis occurs in nucleated duck erythrocytes, but not in human (anucleate) erythrocytes [122, 181, 182]. Immature mammalian erythrocytes (reticulocytes) synthesize this protein, even though these cells are no longer nucleate [88]. However, unlike the mature cells, there remains at this stage an appreciable amount of cytoplasmic RNA.

Although it appears that the nucleus exerts its influence, in part at least, by providing for the synthesis of cytoplasmic RNA, it seems unlikely that nuclear RNA is an actual percursor of cytoplasmic RNA, or at least exclusively so. Barnum and Huseby found that while incorporation of labeled phosphate into the nuclear RNA proceeds at a greater rate than into the cytoplasmic, the ratio of the two rates remained constant during the initial period of incorporation, a fact which does not support the hypothesis that cytoplasmic RNA is derived exclusively from nuclear RNA [17]. Further evidence is given by Crosbie, *et al.* [46], who found differences between the composition of the RNA from the two sources. That synthesis of cytoplasmic RNA might be an autocatalytic process, hence not directly dependent upon a continued synthesis of nuclear RNA, is suggested by Jeener's finding that the synthesis of the former is proportional to the amounts already present [100]. Nevertheless, it seems fairly certain that the production of nuclear RNA is an important governing factor in the synthesis of cytoplasmic RNA. Perhaps a more definite answer to this problem awaits the chemical characterization of the material demonstrated by Pollister, *et al.* [160] and others [74, 166] using electron microscopy, which appears to be traversing the nuclear membrane.

The final stage of this process, the synthesis of protein, has been shown in a number of studies to depend upon the production [147] or, more usually, the presence of RNA [11, 216, 158]. Such a role is also implied by a number of its chemical and physical properties, including that of a proteolytic enzymatic activity [24], possibly the reverse of a normal physiological activity in the cell; and the presence of a highly labile phosphate fraction [14], suggesting that RNA might act as a highly organized energy transfer mechanism, enabling the simultaneous orientation of individual amino acids and synthesis of the peptide bond [61, 186].

5. *Conclusion*

Thus far, the cytophotometric techniques and the *in situ* methods of cytochemical analysis in general have achieved remarkable success in approaching many of the problems of cell growth and development which have not yet lent themselves to the more conventional biochemical methods—this in spite of one rather serious defect, the paucity of methods available for the identification and quantitation of most of the physiologically specific substances of the cell. At present the number of *in situ* methods applicable to the study of specific proteins is rather meager, and the results obtained often, at best, only suggestive. Means for distinguishing between the different "species" of either RNA or DNA do not exist. These shortcomings may no doubt be remedied in the future by the development of new reactions involving, for example, labeled antibodies [43, 44, 175], and by refinements allowing quantitation in such techniques as enzyme localization [53, 89], enabling a more accurate study of specific reactions occurring within individual cells.

Even so, these approaches can hardly touch upon most problems arising at the molecular level: the mechanism of DNA synthesis, protein synthesis, gene action, etc. For analysis of such problems we are dependent upon a more detailed chemical characterization of the substances we stain and measure during the various stages of cell development. Whether such characterization can be carried out through *in situ* methods, using X-ray diffraction techniques and electron microscopy, remains to be seen. In regard to this the future looks quite promising from another angle. It seems probable that techniques such as those devised by Scherbaum and Zeuthen [176] for inducing synchronous division in cells will act as an incentive to the application of the more conventional biochemical approaches to these problems. Using this technique, samples of cells all in the same stage might be drawn from a population at various times during the synchronized growth cycle. In a similar manner, the tissue culture method with its capacity for growing cells with the same genetic background under similar environmental conditions can provide samples of cells which are homogeneous with regard to differential development. The "purification" of the metabolic processes made possible in such systems should go a long way toward providing the biochemist, with his battery of chemical and physical techniques, the homogeneous systems with which he works to such great advantage.

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7. *Pentosenucleic Acids in Relation to Nuclear and Cytoplasmic Functions*[1]

BY JOHN I. NURNBERGER AND
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1. Introduction

THE PENTOSENUCLEIC ACIDS (PNA's)[2] are found in almost all vital systems from the relatively simple plant viruses to the highly differentiated cytoplasm of neurons in the cerebrum of man. It is of more than passing interest that this group of substances, known and reasonably well characterized for almost a century, recognized as a well-nigh universal constituent of living matter for twenty years or more, should, to this day, have been assigned no specific, proven biological role in cellular metabolism. This fact is all the more remarkable when one considers that the chemical and physical characteristics of highly purified representatives of this group have been investigated with exquisitely sensitive tools, that the substances themselves have been accurately localized within living systems very nearly to the molecular level, that their quantitative and even qualitative variations in a bewildering array of physiological and pathological states have been investigated both biochemically and cytochemically. In spite of myriad such studies, their metabolic roles remain speculative or, at best, approximately delimited. ACTH and L-triiodothyronine, a host of steroid hormones, many specific enzymes, glycogen, vitamins, and accessory factors of comparable or greater complexity have betrayed their specific functions after less far-

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[2] The only pentose identified thus far in PNA's is ribose; thus PNA is, for all practical purposes, identical with ribosenucleic acid or RNA. Other abbreviations used are DNA (deoxypentosenucleic acid), ATP (adenosine triphosphate) and AMP (adenosine monophosphate).

ranging search, but the pentosenucleic acids continue to pose a major challenge to biochemists and physiologists. It is tempting to suppose that investigators either have been looking at the right object at the appropriate time and in the correct place but with tools incapable of giving adequately specified information, or have been looking with appropriately sensitive and discriminating tools but only at the fixed and static images of pentosenucleic acid and have thus failed to discover those unique changes associated with biological activity. Such assumptions, however, ignore the complexities of the problem. Some of the tools and techniques which make possible a critical look at this family of substances will be reviewed here, together with relevant data already so accumulated. A number of physiologic and pathologic conditions which offer great promise for revealing fundamental biological activities of pentosenucleic acids will be noted.

The organization of this presentation may seem to be somewhat unconventional in that data concerning the isolation, purification and chemical characterization of pentosenucleic acids are considered first. One might be provoked to ask what such data have to do with the metabolic activities of cellular PNA's. In answer, many recent investigations have shown that only in the light of this crucial knowledge can we evaluate accurately information concerning the distribution, fluctuations, and possible modes of action of these substances.

2. *The Isolation and Chemistry of PNA*

RECENT TECHNIQUES FOR ISOLATING PNA. Accurate information concerning the components and molecular configuration of any constituent of living matter is predicated on the availability of a relatively pure analytical sample which bears substantial resemblances to the material as it exists in its normal functioning environment. For this reason the great importance of satisfactory techniques for isolating PNA's has been stressed in recent reviews [9, 93]. Magasanik [93] has detailed historical landmarks in the solution of problems of PNA isolation and has summarized current trends in isolation techniques. A number of factors create difficulties in isolation. Among these may be mentioned the strong attraction of PNA's for other tissue constituents, the presence within the tissues of depolymerizing and hydrolyzing ribonucleases, the difficulty of separating completely the pentosenucleic acids from deoxypentosenucleic acids, and the relative resistance of most isolated pentosenucleoproteins to dissociative maneuvers designed to give protein-free samples. A further fact of critical importance is the extreme sensitivity of such preparations to slightly alkaline or moderately acid pH.

A number of techniques have been proposed to overcome such obstacles to successful isolation. Thus all recent isolation procedures avoid use of alkali—as urged, in fact, by a few pioneer investigators as far back as 1920 [80]. Effective separation of cellular PNA's from DNA's, not a problem in the isolation of plant virus preparations [28, 29], is usually effected in mixed systems by differentially solubilizing one component in salt solutions of appropriate molar concentration. More serious difficulties have been encountered in reducing or eliminating the activity of tissue ribonucleases during early phases of isolation. Sodium dodecylsulfate [80] and guanidine hydrochloride [66] have both been considered as inhibitors of the ribonucleases as well as deproteinizing agents, though their inhibitory powers have not been satisfactorily demonstrated [93]. Whether citrate itself [80] can be considered a ribonuclease inhibitor is not clear. Doubt has been expressed concerning its efficacy as a DNA-ase inhibitor [144]. Only Bacher and Allen [2] have published convincing evidence that ribonucleases, at least in pancreas, can be semiquantitatively removed or inhibited by initial extraction of tissue homogenate with 50 per cent acetone. By this technique they were able to isolate a pancreatic PNA of substantially lower guanine content than any previously reported.

Complete deproteinization of analytical samples is close to realization. Practically protein-free preparations have been obtained from a variety of animal tissues and from wheat germ by Kay and Dounce [80], using sodium dodecylsulfate. Equally satisfactory preparations have also been obtained by Grinnan and Mosher [66] with the Volkin and Carter guanidine hydrochloride procedure followed by dissociation and removal of protein during prolonged agitation in a mixture of octanol and chloroform. Somewhat less satisfactory samples have been obtained by dissociating isolated nucleoproteins in three molar salt solutions for prolonged periods [81]. Adequate criteria for the presence or absence of protein contamination are now established and have been satisfied by such samples.

Whether or not substances so prepared are faithful representations of *in vivo* metabolites cannot be stated with certainty. Gulland [68] suggested, almost ten years ago, that nucleic acid molecules may become artifactitiously aggregated during the very process of isolation. The degree of so-called polymerization of purified PNA cannot, *prima facie,* be accepted as being directly proportional to its natural condition in biological systems. In this regard, recent observations from this laboratory [64] should be mentioned. The Kay and Dounce technique gave yields of 253 and 270 milligrams of PNA per 100 grams of fresh liver in two separate isolation experiments on comparable groups of control-fed rats. Subsequently the same procedure was followed in all respects except that rat livers were initially blenderized at -20° C in equal parts of acetone and 0.090 molar phosphate buffered to pH 7. The sediments were restirred for 30 minutes in acetone-phosphate at -20° C and thereafter extracted in the usual way [80]. Under the latter circumstances the total yield of PNA was 141 milligrams per 100 grams fresh liver, approximately one-half of previous yields. Analytical data on these samples are available elsewhere [64]. Expected amounts of soluble nucleotides were recovered from the acetone extracts; therefore polynucleotides were not lost in measurable amount during this extraction. This particular procedure was designed to inhibit and/or remove immediately intracellular ribonucleases, and was thought to accomplish this by permitting extractions at relatively low temperatures, augmenting prompt dehydration and removing soluble ribonucleases. These observations are consistent with Gulland's already-mentioned warning and keep alive the possibility that isolated PNA's of high molecular weight prepared by such methods [66, 80] may, in part, be preparatory artifacts.

THE CHEMISTRY AND STRUCTURE OF PNA. In 1947 Gulland [68] posed five unanswered questions about the chemistry of PNA's. These questions concerned molecular size and configuration, the nature of individual nucleotides, the types of internucleotide linkages, the relative proportion of nucleotides, and, finally, the precise sequence of nucleotides in PNA's. It is eloquent testimony to the competence and ingenuity of subsequent investigators that only two of these questions, relating to the precise sequence of nucleotides and molecular size and configuration, now remain a matter of debate.

All known PNA's from animal, plant, and microbiological sources are complex aggregates of the four fundamental nucleotides, adenylic, guanylic, cytidylic, and uridylic acids. Moreover, the specific pentose in all PNA's thus far studied has been identified as ribose in the cyclic ribofuranose form. The only remaining constituent is phosphate, through which the mononucleotides are interlinked. There is very little convincing evidence of any qualitative difference between PNA's, although Bernheimer [5] has suggested possible differences because of the variable capacity of preparations from heterogeneous sources for augmenting or inducing streptolysin formation in streptococci. PNA's isolated from tobacco mosaic virus are inactive in this respect, whereas preparations obtained from wheat germ, tobacco leaves, mammalian liver, yeast cells, and streptococci themselves are quite active. He observed similar variations in the capacity of PNA's from different sources to inhibit extracellular DNA-ase produced in cultures of Group A streptococci. Such specific differences in PNA activity may be related to significant variations in the nucleotide composition or sequence as amplified later.

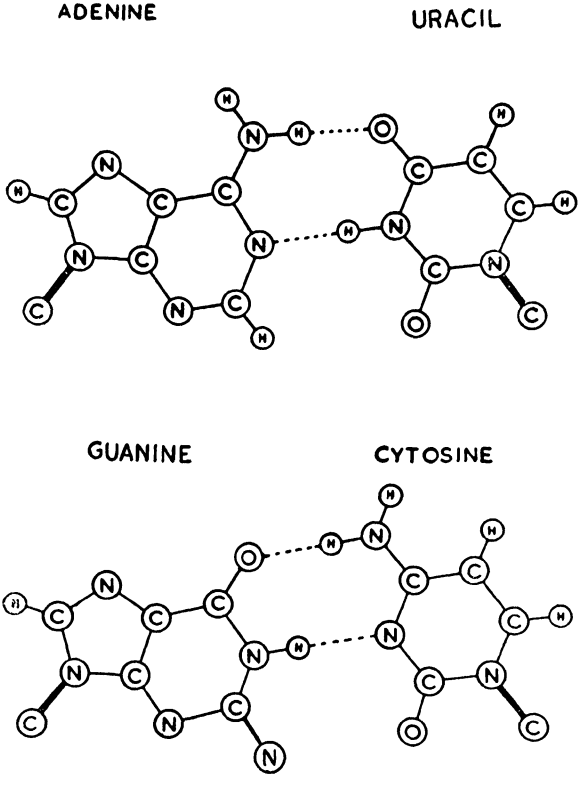
It is generally agreed that the amount of phosphate in a purified preparation of PNA bears some relation to the degree of aggregation of the sample. More highly aggregated products have generally been considered to be more "natural" or "native" probably because of their similarity to macromolecular PNA obtained many years ago from plant viruses [29], where problems of isolation are relatively few. Questions concerning the validity of such an interpretation have already been posed. The phosphorus content of an isolated preparation is sensitive to a variety of factors. Vignais [139] has prepared PNA moieties of increasing phosphorus content by exposing preparations to increasing concentrations of acetic acid. Subsidiary data of Vignais indicate that the higher the phosphorus the lower the degree of aggregation, somewhat analogous to the situation with glycogen. In *in vitro* systems Dounce and Kay [43] and Bresler and Nidzyan [10] record significant transfer of phosphorus from ATP to highly polymerized PNA after incubation with myokinase or rat liver homogenate respectively. The possibility that acid precipitation of Dounce and Kay's postincubation PNA preparations might in itself tend to increase phosphorus content (see Vignais) is worth mentioning. Whether strictly comparable control studies have canceled this factor is not clearly spelled out in the report. The phosphorus content of purified preparations of PNA from various sources ranges from about 7 per cent to 9 per cent or more. Those with lowest values have been isolated by quite mild procedures.

Nearly protein-free preparations of high molecular weight PNA were first isolated from heat-denatured tobacco mosaic viruses in 1942 [29]. These were spontaneously birefringent, a characteristic indicating significant molecular orientation, and had an average particle weight in the neighborhood of 300,000. Similar preparations have recently been obtained from mammalian sources and wheat germ, as already mentioned.

The specific configuration of the constituent nucleotides of PNA has been established through the pioneer work of Brown and Todd [12, 137], Markham, Smith, and associates [96], and Chargaff, Magasanik, and associates from the Columbia group [22, 25, 93]. The commonest internucleotide linkage is 3′:5′ phosphodiester type [12, 92, 95, 96, 137]. Data supporting this conclusion have recently been summarized [137]. The alkaline lability of PNA's and the contrasting stability of preparations of DNA reflect the fact that DNA lacks a hydroxyl group on the C2 position of the ribofuranose chain and therefore is unable to participate in the formation of cyclic 2′:3′ nucleotides which have been demonstrated as intermediates in the alkaline hydrolysis of PNA [12, 96, 137]. Markham, Matthews, and Smith [95] present evidence that there are two different types of PNA's, those with terminal 5′: phosphates and those with terminal 3′: phosphates. Their analytical data suggest that the plant virus PNA's studied are predominantly of the latter type, except for turnip yellow mosaic virus, which is of the former type, together with preparations from yeast. It is certain that they have demonstrated significant differences between preparations from these sources, though their implied suggestion that these PNA's arise from different basic building blocks remains to be demonstrated. It is established [95, 137] that intestinal phosphatase acting upon ribonuclease hydrolysates of PNA yields large amounts of nucleoside-5′-phosphates. Snake venom phosphodiesterase, moreover, is capable of attacking undegraded PNA [123, 124], despite the presence of 6–10 per cent of the phosphates as terminal 3′: phosphoryls, to liberate 5′: nucleoside phosphates. This fact is the basis for the continuing reservations of Schmidt concerning an exclusive 3′:5′ internucleotide link [123].

Branching of polynucleotide chains is now considered highly probable [12, 93, 137], and Brown and Todd indicate [12] that both linear and branched chains of polynucleotides may well coexist. Both phosphodiester and phosphotriester branch linkages have been proposed, the former being of C3′–C2′ type. Branching is possibly of profound importance in reference to the natural state and permutations of native PNA's. Some physicochemical evidence of branching in the linearly arranged DNA molecules has been published but is not supported by chemical data as such [113].

Up to the present there have been relatively few studies with any precise bearing on nucleotide sequences in the PNA molecule. Techniques for investigating this problem have been carefully reviewed [12, 136]. A study of Schmidt and his co-workers [124] some years ago on the enzymatic hydrolysis of PNA's with production of the now generally accepted purine-rich core was considered incompatible with the hypothesis that there is a regular alternating sequence of purine and pyrimidine groups. As a matter of fact, the known nucleotide and base compositions of a wide variety of PNA's make such a regular alternating sequence patently impossible.

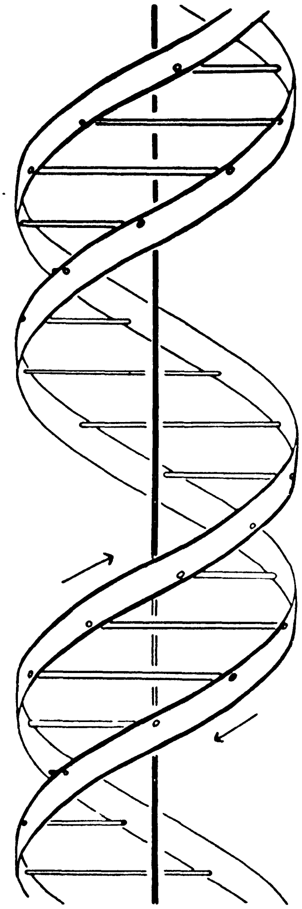


TEXT FIGURE A. Hydrogen-bonding as visualized for the base core of current models of macromolecular PNA's. Above: bonding between adenylic and uridylic nucleotides; below: bonding between guanylic and cytidylic nucleotides. Circled C's in all formulae, connected by heavy set lines, represent the first carbons of the attached ribofuranose rings. Phosphates not shown.

The nature of aggregation of polynucleotide chains into macromolecules is still matter for conjecture. On a purely physical level Vandendriesche [138], a number of years ago, noted an initial volume increase followed by a subsequent volume contraction during hydrolysis of yeast

PNA with pancreatic ribonuclease. The contraction noted bore a linear relation to the liberation of acid groups. Recent studies of related type [113] indicate that at least DNA, which is structurally analogous to PNA in some respects, behaves like a cross-linked, coiled polynucleotide which is under considerable restraint, probably because of the small number of branching or network points. Vignais [140, 141] has stressed the importance of secondary bonds of hydrogen-bridge type contributed by the pyrimidine and purine base components in the formation of highly polymerized PNA's. The type of bonding shown in Text Figure A for the appropriate base components of PNA is a fundamental part of the macromolecular structural configuration proposed by Rich and Watson [115]. These workers visualize a two-stranded helical structure in which component chains are held together by such hydrogen-bonding effects. Pauling and Corey suggested such a helical arrangement of polynucleotide chains. In their early model of PNA [108], which has since been discarded by them, three such helices were spatially oriented around a phosphate core. An analogy to the structure of glycogen provided precedent for this structure. Jordan recently summarized critical thinking [79] on the model of Pauling and Corey and mentions not only that the disposition of phosphates in the core is at variance with the behavior of nucleates in solution but also, quoting Watson and Crick, that many of the Van der Waals distances in this model are too small. Pauling currently favors an RNA model analogous to that for DNA proposed by Watson and Crick [143], which is reproduced as Text Figure B. Such a structure as this provides favorable configuration for chain branching, which, as previously mentioned, seemed increasingly probable especially for PNA's. A definitive solution of the complex problem of macromolecular structure of PNA's awaits the preparation of samples more satisfactory for X-ray diffraction study.

NUCLEOTIDE AND BASE COMPONENTS OF PNA'S. It is generally thought that the nature and order of the individual nucleotides in PNA's confer certain specificities on these substances. The only pentose sugar thus far identified in purified PNA is, as already mentioned, d-ribose. The clarification of the types and ratios of constituent nucleotides has, therefore, been a work of major importance. The advent of paper and column chromotographic techniques in recent years has permitted intensive analysis of samples from widely divergent sources and has furnished data of unparalleled qualitative and quantitative reliability. The striking regularities noted for isolated DNA's serve as an interesting point of departure in a discussion of PNA's. Chargaff [23] summarizes analytical data obtained on DNA's from many different sources and distinguishes two principal groups of compounds, those of the so-called "AT" type in which adenine and thymine are the dominant bases and those of the "GC" type in which guanine and cytosine dominate. The DNA of *E. coli* is a representative of an intermediate group in which the bases are present in near equimolar proportions. In addition to these bases, 5-methylcytosine [23, 147] and hydroxymethylcytosine [145] have been identified as components of DNA's, the latter from



TEXT FIGURE B. Diagrammatic representation of the spatial orientation of two helical polynucleotide chains in DNA. The two ribbons symbolize the phosphate-sugar chains and the horizontal rods, the paths of bases holding the chain together. The vertical line marks the fiber axis. From Watson and Crick [143], Fig. 4.

TABLE 1. Nucleotide Composition of Pentose Nucleic Acids; Molar Relationships

Preparation No.	*Source*	*Guanylic Acid*	*Adenylic Acid*	*Cytidylic Acid*	*Uridylic Acid*	*Purines: Pyrimidines*
1	Yeast	9.7	10	6.1	7.0	1.5
2	"	9.6	10	7.5	6.7	1.4
3	"	10.6	10	8.6	8.2	1.2
4	"	10.5	10	8.0	10.2	1.1
5	Pig pancreas	22.5	10	9.8	4.6	2.3
6	" liver	16.3	10	16.1	7.7	1.1
7	Sheep liver	16.8	10	13.4	5.6	1.4
8	Calf "	16.2	10	11.1	5.3	1.6
9	Beef "	14.6	10	10.9	6.6	1.4
	Carcinomatous human liver					
10	Unaffected tissue	32.9	10	28.8	8.3	1.1
11	Metastases	41.4	10	43.2	7.2	1.0

Molar ratios are referred to adenylic acid as 10. From Chargaff and co-workers [25], Table IV. For details of analytical procedures see that reference.

certain bacteriophage nucleic acids, the former from DNA's of calf thymus, wheat germ, and a number of other sources. These are either trace components or, in some instances, total replacements for cytosine. Fundamental general and species regularities in all DNA's studied are established [21, 23, 26, 31], since the molar ratios of adenine to thymine, guanine to cytosine, total purines to pyrimidines, and total 6-amino to 6-keto groups are all equal to 1.00 with variations in the third decimal place. The possibility that one may be dealing with a large number of differently constituted individual macromolecules covering a broad spectrum of structural gradations even with the DNA's has been mentioned by Chargaff and his associates [24]. They suggest that the fractional dissociation of such substances from protein to give many different DNA's may be dependent on differences in stability of the bonds linking the deoxyribosenucleic acids to proteins.

The only bases thus far identified in PNA's from a wide variety of plant and animal sources are adenine, guanine, cytosine, and uracil. Analyses even at the single-cell level in the highly specialized neuron [47] reveal no other components with respect to PNA nucleotides. The nucleotide composition of PNA's from several animal sources are compared with analyses of yeast PNA in a published study of Chargaff and associates [25], from which Table 1 is reproduced. The composition of liver PNA's shows a certain regularity broadly borne out by other published studies [22, 64, 93, 97]. Similar base ratios have been reported for mammalian brain PNA [41] and for that of carp muscle as mentioned by Magasanik [93]. Within this relatively limited group the PNA's might be characterized as of "GC type," with guanylic and cytidylic nucleotides dominating. It is worth mentioning that the DNA's isolated from comparable sources are usually of "AT type." This possible complementarity has been noted by Magasanik [93] and must prove attractive to those who have a natural bent for manipulating template concepts.

Increasing attention to the physiologic state of the PNA source and more rigid standardization of gentle isolation procedures give resultant PNA's of more similar composition [50, 51, 64]. It seems highly improbable, however, that the various PNA's can ever be reduced to two such simple structural families as the adenine-uracil and guanine-cytosine types characteristic of DNA's [21, 23]. PNA's isolated from tobacco mosaic viruses generally show a significant preponderance of adenine and a very slight preponderance of uracil over guanine and cytosine (see Table 2), but there appear to be many gradations even within PNA's from strains of the same type of virus and from yeast (Table 1 and [130]).

Remarkably little attention has been directed to the influence of changing physiologic states on the nucleotide components of isolated PNA's, in spite of the fact that marked fluctuations in nucleotide ratios have been observed in preparations from seemingly identical or similar sources. Elson and Chargaff [50] determined the nucleotide composition of PNA's isolated from normal and lithium-treated sea urchin eggs and embryos and found no significant variations in molar ratios in PNA's from unfertilized eggs and from developing embryos during the first forty-eight hours of morphogenesis. The adenylic:guanylic:cytidylic:uridylic ratios were very close to 10:12:12.5:8 throughout. Gold and Sturgis [62] report significant differences in purine and pyrimidine ratios for PNA's isolated from uterus, liver, and spleen of rats maintained on widely varying levels of steroid sex hormones. They find a gradual decrease in the guanine and an increase in the adenine content of the PNA of endometrium during the shift from proliferative through late secretory and finally menstrual phases. Their data are difficult to interpret largely because of the highly complex physiologic system with

TABLE 2. Proportions of Purines and Pyrimidines in Pentose Nucleic Acids Obtained from Some Strains of Tobacco Mosaic Virus

Strain	*Preparation No.*	*Molar Proportions Calculated to Total 4* *Adenine*	*Guanine*	*Cytosine*	*Uracil*
TMV	1	1.16	1.01	0.75	1.05
"	2	1.17	1.02	0.74	1.06
"	3	1.20	1.02	0.73	1.04
"	4	1.21 (1.19)	0.98 (1.01)	0.75 (0.74)	1.05 (1.05)
M	1	1.17	1.02	0.79	1.04
"	2	1.18 (1.18)	1.07 (1.05)	0.74 (0.77)	1.02 (1.03)
J14D1	1	1.18	1.01	0.76	1.06
"	2	1.21 (1.20)	1.00 (1.01)	0.72 (0.74)	1.08 (1.07)
GA	1	1.15	1.03	0.76	1.06
"	2	1.19 (1.17)	1.05 (1.04)	0.77 (0.77)	1.01 (1.04)
YA	1	1.19	1.03	0.75	1.04
"	2	1.18 (1.19)	1.01 (1.02)	0.73 (0.74)	1.07 (1.06)
HR	1	1.14	1.08	0.70	1.10
"	2	1.20	1.02	0.74	1.04
"	3	1.17 (1.17)	1.00 (1.03)	0.72 (0.72)	1.10 (1.08)
CV3	1	1.03	1.03	0.73	1.21
"	2	1.02 (1.03)	1.01 (1.02)	0.72 (0.73)	1.24 (1.23)
CV4	1	1.05	1.02	0.76	1.17
"	2	1.01 (1.03)	1.03 (1.03)	0.77 (0.77)	1.19 (1.18)

The figures in parentheses are the averages of those in the respective groups. Each value throughout the table represents the average of three or four replicate analyses of a given preparation. From Knight [84], Table I.

which they are dealing. Very recently it has been observed [64] that the PNA's isolated from control-fed rat liver by the Kay and Dounce technique show a consistent adenine:guanine:cytosine:uracil molar relationship of 10:15:17:11, whereas the PNA's isolated from litter-mate-fed animals after one hour of cold exposure show, by contrast, marked increases in the relative amounts of guanine and cytosine. Such striking shifts in base ratios of isolated PNA's from the same source under carefully controlled experimental conditions suggest a marked physiologic lability of these substances. Changes such as these provide an encouraging clue to an understanding of at least one physiologic role of PNA's in actively metabolizing tissues.

Comparatively little published information bears on the important question of whether the composition of purified and isolated PNA's faithfully reflects that of native substances within the tissue. It is encouraging to note, however, that cytoplasmic and whole cell PNA's from sucrose and citric acid liver homogenates analyzed by Elson and Chargaff [51] were very nearly identical in composition to purified preparations of liver PNA studied by them and many others. In more direct support of the inference that isolated preparations are representative of *in vivo* materials, Knight [84] reports that the base ratios of intact tobacco mosaic virus nucleoproteins are almost identical with those of PNA's isolated and highly purified from the same sources. However, because the procedures for isolating PNA's, especially from mammalian sources, generally yield not over 50 per cent of the known total PNA, it is evident that biochemists must demonstrate, in each instance, that their purified preparations bear a recognized relation to naturally occurring materials.

A number of investigators [37, 50, 51, 97] have shown, beyond reasonable doubt, that the composition of PNA's from the nuclei of mammalian cells differs quite significantly from that of cytoplasmic PNA's. No conspicuous regularities have been noted for the former preparations. The fact, however, that such variations exist is of particular interest in a consideration of the different metabolic potentialities of these two groups of substances, as will be mentioned later.

THE NATURE OF PROTEIN IN THE PRESUMED PNA-PROTEIN COMPLEX. Almost all workers tacitly assume that in living systems cellular pentosenucleic acids exist in intimate combination with proteins. Magasanik cautions [93], however, that the polar groups on protein molecules and PNA's are particularly well suited for creating mutual attractions serving to bind the one to the other. For this reason he emphasizes that free PNA could not be found in tissue extracts in the presence of reactive cytoplasmic protein even though it might exist in that state in the intact cell. He concludes that nucleoproteins customarily isolated from cytoplasmic extracts should not be considered characteristic components of the intact cell without further evidence. The plant viruses have been particularly valuable nucleic-acid-rich systems to study for elucidating this problem. Watson [142] has recently suggested, on the basis of X-ray diffraction evidence, that the tobacco mosaic virus particle (ribosenucleoprotein) has a helical structure consistent with a configuration in which nucleic acid molecules are located in the interior surrounded externally by the protein component of the molecular aggregate. A brief word is in order here concerning DNA-protein complexes. Chargaff [20] some years ago suggested that protein in association with DNA may, in some instances, be its own depolymerase but cautioned that the isolated nucleoprotein may not represent a true cellular constituent, as already mentioned for PNA. Fraser and Jerrel [56] have published data on the amino acid composition of T3 bacteriophage protein which they noted to be remarkably constant under a variety of conditions including alterations in the carbon and nitrogen sources of the host bacterium. They summarized provocative observations of Hershey and Chase which indicate that the basic reproducing unit of such bacterial viruses may not be a nucleoprotein complex at all but rather the nucleic acid fraction. Those workers observed that on infection the sulfur-containing virus protein remains outside the host cell. The nucleic acid component, once it has entered the host, can be isolated from its protein complex without altering its reproductive properties, as they noted. This certainly suggests that under certain circumstances in living forms the DNA-protein complex, at least, may be a highly labile one. No comparable studies have been published for any PNA's. Where specific properties, such as infectivity, have been investigated, PNA-protein complexes have always been involved. Ginoza and his associates [60] have succeeded in removing a colored material from tobacco mosaic virus which contains approximately 35 per cent PNA and 70 per cent protein in contrast with the original virus, which contains about 6 per cent PNA and 94 per cent protein. The residual decolorized material retains its infective properties but still contains protein.

PNA's have been isolated from a variety of cellular sources [110, 133] and their electrophoretic mobilities determined. Even in particulates which do not sediment at 60,000 × g., PNA's still exist in stable complex with protein. Cohen [28] stated in 1947 that the links binding plant virus PNA to its protein were not of the salt type, though more recently Riley and Arndt [116] could find no physical evidence for anything except a salt-bridge type of union in herring sperm and calf thymus protein complexes with DNA, which are probably similar to those with PNA. Specific types of protein have been identified with PNA-rich particulates of cytoplasm, though there is no convincing evidence that they exist in intimate complexes. McShan and his associates [100] found that pituitary gonadotropin was concentrated in a fraction which was probably predominantly microsomal. If this is a natural nucleoprotein complex, it represents one of exquisitely specific type. Novikoff's [104] recent studies on the subfractionation of so-called microsomal particulates have demonstrated a marked chemical as well as enzymatic heterogeneity of fragments isolated at varying gravitational force. The more readily sedimented microsomes which were rich in PNA showed a high esterase, adenosine 5′-phosphatase, acid phosphatase, and uricase activity, while less readily sedimented microsomes, still very rich in PNA, had low levels of acid phosphatase and uricase activity. The implication is, of course, that at least in liver no one specific protein or class of proteins forms complexes with PNA. The very good possibility should be considered that the cellular particulates defined as microsomes are themselves partly fragmentation artifacts of a highly complex cytoplasmic system which is morphologically distinct. Palay and Palade [106] in their recent electron microscopic study of neuronal cytoplasm find no cytoplasmic structures of a size and shape analogous to any except the smallest sedimentable microsomes, i.e. those in the neighborhood of 25 millimicra. Herrmann and Nicholas [71], in their study of the myosin of rat muscle during development, did not imply that this protein could or did exist in complex with PNA (in fact, quite the contrary), but the very interesting observations of Hamoir [70] do indicate that nucleoprotein complexes from muscle can be isolated, highly purified, and, in fact, crystallized. Hamoir succeeded in isolating two crystalline proteins from fish muscle, both showing the solubility properties of tropomyosin and both containing PNA, one 10 per cent or more and the other 3 per cent or less. However, this investigator cautions that further work is necessary to prove that such complexes exist in normal muscle, whatever the crystalline properties of the isolated product may be. Therefore, the usually accepted interpretation that PNA's exist at the cellular level in complexes of varying intimacy with proteins is supported by much inferential evidence but by little conclusive objective evidence. The whole concept of a structurally specific PNA associated with a structurally specific protein is most attractive to the "template" enthusiasts, but the concept is still strictly hypothetical. However, techniques are now available for exploring this very stimulating hypothesis.

THE CELLULAR DISTRIBUTION OF PNA'S AND CLOSELY RELATED SUBSTANCES. As mentioned in the introduction, nucleic acids constitute a conspicuous component of "living"—self-duplicating or self-duplicated—systems. Bacterial viruses [28, 65] contain only DNA and protein material. Plant viruses, in contrast, contain only PNA and protein, the former constituting up to 40 per cent of total material. Many animal viruses contain both forms of nucleic acids with protein. Mention has already been made of the proposed interrelationships of tobacco mosaic virus PNA and protein. Such virus proteins as have been analyzed have usual amino acid components. It should be mentioned [65] that few, if any, enzyme systems have been demonstrated in viruses. In this sense such particles differ quite substantially from all except the smallest sedimented mammalian microsomal components, which have on occasion been likened to virus particles because of superficial resemblances. Extensive cytochemical studies in bacteria have been published [18, 94] and Malmgren and Hedén describe two types of nucleic acid systems in gram-positive bacteria. A DNA system which appeared to be organized in a structure analogous to the cellular nucleus was likened by them to the euchromatic system of more organized cells, while the PNA system contained in the outer layers of such bacterial organisms was considered the counterpart of the heterochromatic system of mammalian cells. Certain metabolic potentialities of the PNA of such living systems will be mentioned in the next section.

Caspersson, in a series of publications [16, 17, 18], has summarized cytochemical evidence for the occurrence and distribution of PNA's both in the nucleus and cytoplasm of a variety of different plant and animal cells. He has drawn sharp distinctions between the PNA occurring in the nucleus and that present in the cytoplasm, particularly as regards the presumed sequence of metabolic events in cellular division and protein synthesis. Bensley and Gersh [3] wrote an extensive review on the distribution of basophilic substances in a variety of cells with particular attention to the so-called Nissl substance of the neuron. Since that time cytoplasmic basophilia has been convincingly shown to indicate the presence of pentosenucleic acids, though more selective stains than toluidine blue, for example pyronine, have been recommended for their demonstration [8]. Protease-free ribonucleases have been widely used to aid in distinguishing between cellular DNA's and PNA's in cytochemical studies. In Figures 57–61, the distribution of cellular nucleic acids is demonstrated. Figure 57, from Caspersson [16], highlights the distribution of heavily absorbing DNA in the bands of a chromosomal segment from Chironomus as demonstrated by ultraviolet photography. In Figure 58, the distribution of both nuclear and cytoplasmic material absorbing at 257 millimicra in the ultraviolet is demonstrated in frozen-dried sections of rat liver. Figure 59 is a photograph of a section from the same frozen-dried block following ribonuclease digestion for one hour. This demonstrates the almost complete disappearance of particulate absorption in the cytoplasm, with, however, no significant change in absorption of the nuclei of these cells. Relatively large amounts of DNA are, of course, present in the latter. Figure 60 demonstrates the distribution of cellular absorbing materials in frozen-dried sections of the paraventricular and supraoptic nuclei of the rat. A comparable post-ribonuclease digestion picture is shown in Figure 61. The qualitative specificity of this technique for the demonstration of the cellular localization of the nucleic acids compares favorably with that for highly selective staining techniques. From these particular photomicrographs the spatial distribution of cytoplasmic absorbing materials would appear to be quite different in the neuronal elements represented and in liver cells. The fact is, however, that all gradations of microscopic aggregation from the massed and circumscribed clumps of liver cells to the finely dispersed and scarcely resolved aggregates of the neurons can be detected in representatives of each cell-type. Palay and Palade [106] have recently published a highly informative study on the ultrastructure of components of neuronal cytoplasmic zones rich in PNA. Their conclusion, drawn from electron microscopic studies, is that the structural organization of the differentiated cytoplasm of a variety of cells, for example pancreatic, hepatic, and neuronal cells, is fundamentally the same. This cytoplasmic organization, which they consider to be ergastoplasm, is composed of an interlacing endoplasmic reticulum disposed in anastomosing tiers, along the outer surfaces of which they find punctate or rodlike particles from 10 to 30 millimicra in diameter, some of these appearing in clusters in the adjacent ground substance of the cytoplasm. These particles approach in size the calculated dimensions of the smallest sedimented liver particulates identified by Novikoff and his associates [104] in sucrose homogenates of liver. The presence of selective ultraviolet absorbing material in both the nucleus and cytoplasm of living cells is recognized and the considerable alterations in such absorbent materials following fixation, contraction, or dispersion have been studied [101].

The fractional sedimentation of disintegrated or homogenized tissue preparations has furnished data which generally confirm the distribution of PNA-rich components in both the nucleus and cytoplasm as defined by cytochemical studies. Homogenates of highly specialized and locally differentiated tissues such as brain may give data impossible to evaluate in terms of cytochemical data [12], a fact which is occasionally ignored [121]. A critical review of such analyses for the components of the cytoplasm has recently been published by Hogeboom and Schneider [73]. In general, they emphasize the high PNA content of the isolated microsomes from a variety of sources but caution that this is not uniformly true, since in tissues such as adult kidney and spleen, and in embryonic tissues in general, the PNA is mainly associated with fractions which do not have the usual sedimentation characteristics of microsomes. The sedimentation characteristics of PNA-rich microsomes even in adult liver can be altered quite substantially by relatively insignificant changes in physiologic state of the organism. Thus it has been shown [63] that a substantial percentage of the identifiable microsomes of liver and brain cortex sucrose homogenates in rats cold-exposed briefly are not sedimented at 23,000 × g., though supernates from the homogenates of control animal organs are free of recognizable microsomes under the same sedimenting conditions. In view of the relatively broad spectrum of probable microsomal particle sizes suggested by Novikoff's studies [103], labile shifts in sedimenting characteristics are not entirely unexpected. Fragmentation and aggregational artifacts may complicate this picture, as already mentioned. In mammalian cells centrifugal sedimentation data generally reveal that 5 to 15 per cent of total PNA-phosphorus (PNA-P) can be recovered from nuclear fractions, depending upon their purity; 10 to 20 per cent from mitochondrial fractions; 40 to 55 per cent from microsomal fractions; and the remainder from nonparticulate supernates depending upon the individual technique, care in isolation, experimental conditions, and organ source. The ultrastructural disposition of the PNA recovered from mitochondrial fractions has not thus far been clarified. Grinnan and Mosher [66] found that their "highly polymerized" PNA was localized almost exclusively in the microsomes of liver preparations. Since conventional fractionation techniques such as they use do not effect a clean separation of small mitochondria from large microsomes, the significance of this observation awaits further clarification.

Several groups have investigated the distribution and characteristics of cellular ribonucleases [40, 103, 123]. The existence of two enzyme systems is suggested, one active at a pH in the neighborhood of 8.2, and the other maximally active at about 5.8. The latter occurs in pancreas at an activity level far exceeding that of any other animal tissue studied. Though pancreatic ribonucleases are generally considered prototypical and are used for most cytochemical enzyme studies they are not proved to be representative of such activities in other tissues [40, 123]. It is of some interest that cell fractionation experiments indicate that both the alkaline and acid activities are concentrated conspicuously in the mitochondrial fraction of liver though immunological studies referred to by Schmidt [123] do not confirm such a distribution for the acid ribonuclease of pancreas. It is, of course, possible that the conditions under which such enzymatic activities appear optimal in *in vitro* systems differ very substantially from those operative in the cell; thus such studies may not accurately reflect the facts of cellular organization.

Leslie has recently published an encyclopedic review [87] of the distribution of both PNA-P and DNA-P in a wide selection of tissues and microbiological forms under various conditions. In broadest outline, these chemical analyses generally confirm studies at the cytochemical and histochemical level.

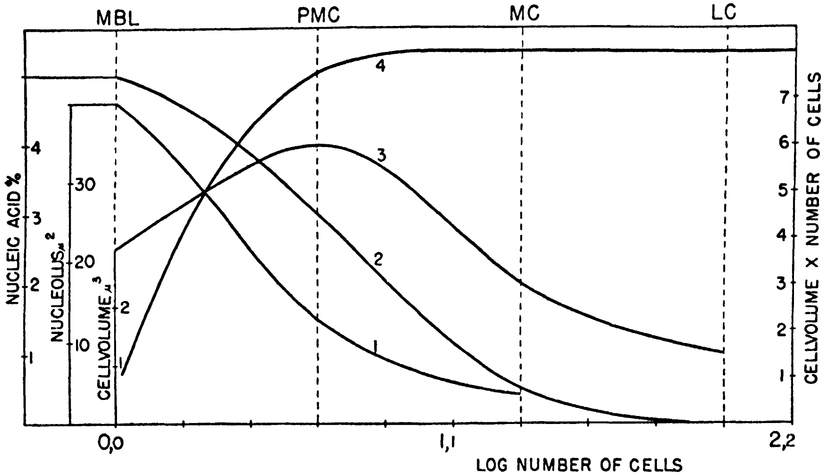
THE OCCURRENCE OF SOLUBLE MONONUCLEOTIDES IN TISSUE EXTRACTS. Kornberg in 1951 [86] summarized the evidence of previous workers as well as his own for the identification and possible significance of the co-enzyme uridine diphosphate glucose (UDPG) originally isolated from yeast cells and demonstrated subsequently in a variety of animal tissue cells and in penicillin-inhibited staphylococcus aureus. In the last named the sugar is replaced by another reducing group sometimes bound to certain amino acids. He was able to separate uridine diphosphate (UDP) from this compound by mild alkaline hydrolysis and also demonstrated the conversion of UDP to uridine triphosphate (UTP) in the presence of pyruvate phosphokinase utilizing phosphopyruvate as the phosphate donor. The resultant UTP phosphorylated glucose and fructose-6′-phosphate with yeast hexokinases although at very much lower rates than the corresponding adenosine triphosphate (ATP). At the time, he postulated that UTP might possibly serve in the synthesis of UDPG from UTP and glucose-1-phosphate or on the other hand that it might, by condensation with ATP, serve a role in the synthesis of polynucleotides. Subsequently Smith and Mills [129] found uridine monophosphate, diphosphate, triphosphate, and the original diphosphate glucose in liver. They showed that not only UDPG but also uridine diphosphate acetyl-glucosamine undergo a pyrophosphorolytic split to yield UTP in the presence of liver nuclei isolated from sucrose media. They discovered enzymatic activities in liver nuclei which caused the breakdown of UTP to UDP, and uridine diphosphate acetyl-glucosamine to UDP, UMP, and uridine. At about the same time, Schmitz and associates [125] isolated and identified the 5′-phosphates (mono-, di-, and tri-) of uridine, cytidine, and guanosine from tissue extracts of liver, muscle, brain, and experimental carcinoma in rats. This important work indicates that all of the nucleotides which occur in PNA exist in the acid soluble fraction of such animal tissues as 5′-phosphate nucleosides. Perhaps more importantly, they exist at three levels of phosphorylation previously established only for the adenine and uridine nucleotides. The demonstration of these constituent nucleotides in cellular extracts is of particular interest not only because of their potential significance as available precursors for PNA but also because of their possible function as energy transmitters analogous to the adenosine pyrophosphates. These concepts will be discussed in more detail shortly.

3. PNA in Cellular Metabolism

Available information about the possible roles played by the PNA's in cellular metabolism derives from two general modes of analysis, the one cytochemical and the other chemical. Cytochemical studies have utilized either radiant energy microabsorption techniques which depend upon the selective ultraviolet absorption of constituent purine and pyrimidine chromophores or on staining reactions specific for PNA. In most published chemical studies PNA is estimated in terms of its constituent phosphate or reducing sugar, largely the former. A relatively few have been concerned with the base or nucleotide components themselves. Chemists have utilized cell disintegration and fractionation techniques by high-speed differential centrifugation. In many instances the tracking of labeled compounds, particularly phosphates or compounds containing labeled carbon or nitrogen, has yielded information of great importance. Data from these various sources will be considered.

CYTOCHEMICAL STUDIES. Over twenty years ago Brachet began a series of experiments on the metabolic role of nucleic acids in morphogenesis. In recent years he and his associates have ranged widely in their study of the metabolic role particularly of PNA in a wide variety of organisms and tissues. These studies have been summarized and evaluated on several occasions [8, 9]. Their conclusions become increasingly conservative but nonetheless clearly indicate that the link between the localized synthesis of PNA and the process of embryonic development and specific morphogenesis is quite close, so close, in fact, that morphogenesis cannot be dissociated from such synthetic mechanisms. He has placed great stress on the existence of PNA gradients and their relationship to embryonic development and differentiation. Brachet's original hope that the evocating action of PNA or its breakdown products might be of critical importance during neural induction has not been convincingly justified, as he emphasizes, since any agent or activity which produces sublethal cytolysis seems capable of producing such inductions. The assumption that PNA or more specifically cytoplasmic microsomes —which he has likened to "self-duplicating," virus-like particles—play an important part in morphogenesis is not too well documented. Moreover, the fluctuations in proportion of sedimentable particles rich in PNA from unfertilized eggs as compared with differentiating and adult tissues may not be so unique a characteristic of morphogenesis, since such variations in sedimentation characteristics of microsomal aggregates have been demonstrated [63] in adult tissues, as already mentioned.

Caspersson and his associates have utilized his ultraviolet microabsorption techniques extensively in a comprehensive study of the absorptive capacity of cellular components under a variety of conditions. Thorell [134, 135] has correlated cytoplasmic ultraviolet absorptive capacity with morphological change during granulocytopoiesis. Essential data from these studies are presented diagrammatically in Text Figure C. From this diagram it is evident that the cytoplasmic ultraviolet absorptive capacity (recorded as "Nucleic Acid %") as well as the nucleolar cross-sectional areas decrease quite rapidly at a time when cell volume is increasing. His data suggest an inverse relationship between the degree of specific ultraviolet absorptive capacity in these cells and their transition from stem cells to mature or maturing granulocytes. The precise interrelationships are, however, not simple, since cytoplasmic ultraviolet absorptive capacity decreases progressively from an extremely high level in the cytoplasm of myeloblasts to the relatively low level in more mature cells of the granulocytic series, whereas cell volume reaches a maximum in the promyelocyte. These data are, of course, entirely consistent with the idea that the least differentiated cell and therefore the one with the greatest differentiating potential shows a high degree of cytoplasmic absorptive capacity. However, the process of growth and differentiation itself appears to be correlated with rapidly decreasing cytoplasmic absorptive capacity. Of perhaps even more interest are Thorell's observations [135] on the morphological differentiation and appearance of hemoglobin in the maturing red blood cell, summarized

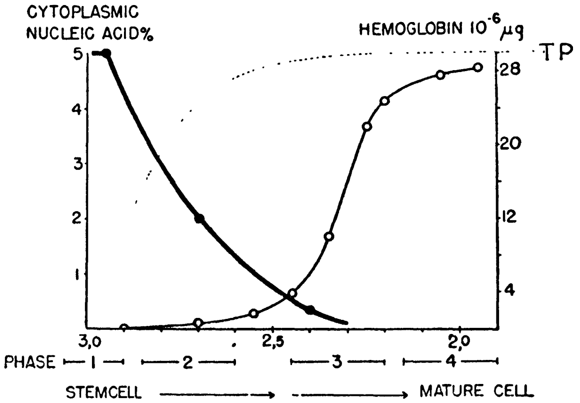


TEXT FIGURE C. Survey of some essential cytochemical and cytological changes connected with the endocellular growth processes during granulocytopoiesis. MBL = myeoblast, PMC = promyelocyte, MC = myelocyte, and LC = mature granulocyte. The curves are: 1, optical cross section area of the nucleoli in the cell; 2, cytoplasmic ribose polynucleotides in per cent; 3, volume of the cell; 4, total volume of cells in each developmental phase.

From the diagram it appears that before and during the principal increase in cellular mass (about 8×) the granulopoietic cells are characterized by a large nucleolar mass and high concentrations of ribose polynucleotides in the cytoplasm. During the course of maturation the nucleolar mass and the cytoplasmic ribose polynucleotides decrease parallel with a cessation of the growth activity of the cell.

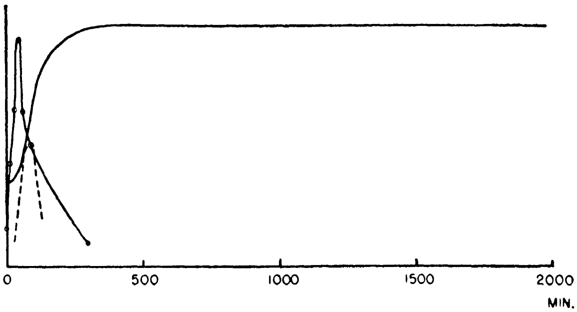
in Text Figure D. Again, the ultraviolet absorptive capacity of the undifferentiated stem cell cytoplasm (cytoplasmic nucleic acid percentage) is relatively quite high and falls rapidly as the total cellular protein rises. The endocellular elaboration of the specific protein, hemoglobin, is, however, not related in any precise way to measurable shifts of cytoplasmic absorptive capacity or presumed polynucleotide concentration. Both of these studies are somewhat difficult to interpret on a metabolic level, since concentration data—or, more specifically, cytoplasmic absorptive capacities—are compared with total cell volumes. What is demonstrated is that as primitive stem cells differentiate and multiply, the resultant more mature cells have progressively less capacity to absorb ultraviolet light selectively.

Hydén and his co-workers have published a series of cytochemical studies on the specific ultraviolet absorptive capacity of nervous tissue during development and functional activity [69, 76, 77]. They noted, as did Thorell, that the specific ultraviolet absorptive capacity of the neuroblast or stem cell increases markedly at a time when differentiation of this cell into the adult type begins to be manifest. They laid considerable emphasis on the presumed role of the heterochromatin of the nucleus in production of nucleolar proteins rich in so-called "hexone bases" with subsequent accumulation of such PNA-protein complexes at the nuclear membrane and diffusion into the immediately adjacent cytoplasm. The appearance of heavily absorbing zones in the region of the nuclear membrane during the process of differentiation and division was associated by them with the extensive protein synthesis accompany-



TEXT FIGURE D. Survey of the essential changes in cytoplasmic composition during erythropoiesis. Abscissa: logarithm of cellular volume; —•—•— cytoplasmic ribose polynucleotides in per cent; —o—o— total amount of hemoglobin in the cell, calculated from the absorption measurements in the Soret-band. TP, total cellular protein obtained from the total volume of cells in each developmental phase. The figure shows, on the whole, that before the erythrocyte is differentiated into its final functional stage, the ribose polynucleotide metabolism associated with the endocellular growth processes is finished, i.e. that during the unipotent cell development the new formation of the cellular protein substances is completed before differentiation occurs into its final, specific form. From Thorell [135], Fig. 48.

ing such multiplication, growth, and differentiation. Hydén and his associates recorded quite rapid fluctuations in the ultraviolet absorptive capacity of adult motor neurons during fatiguing exercise and also in sensory neurons following electrical stimulation. They observed an initial increase in the ultraviolet absorptive capacity of the cytoplasm of such cells following brief or mild excitation. After more intense stimulation a gradual and ultimately marked decrease in absorptive capacity was reported.



TEXT FIGURE E. E. coli growth and nucleotide curves. Continuous curve = logarithm of the number of bacteria per ml. Continuous curve with rings = nucleotide content of the individual bacterium. The broken curve $= \frac{dn}{n \cdot dt}$ n = number of bacteria per ml. From Malmgren and Hedén [94], Fig. 7.

Malmgren and Hedén [94] have followed variations in ultraviolet absorptive capacity of a number of gram negative and gram positive bacteria during various phases of growth and multiplication. Text Figure E, from their study, indicates the interrelationship between quantitated changes in absorptive capacity and the logarithmic growth curve for this organism. They did not distinguish between PNA's and DNA's in this particular study, but chemical analyses recently summarized by Leslie [87] indicate a rise in the PNA to DNA ratio during such rapid growth phases. Recent experiments of Chayen and co-workers [27] cast a new and particularly revealing light on the observations of Malmgren and Hedén. They have identified both a labile polyphosphate material as well as nucleic acid in trichloracetic acid hydrolysates of Schneider fractions from *Torulopsis utilis.* During active growth of this organism total polyphosphate decreases, but it rises after protein synthesis has terminated. The ultraviolet absorptive capacity of the nucleic acid of this form, calculated per unit of nucleic acid phosphorus (NAP), is found to vary quite substantially during the rapid growth phase. This value reaches a maximum at the termination of rapid protein synthesis but is actually at a minimum during early growth. It is possible, therefore, that shifts such as Malmgren and Hedén have observed reflect in part qualitative rather than quantitative alterations in constituent nucleic acids.

Caspersson [18] has recently reviewed many of the studies of his group which support the thesis that the nucleic acids of deoxy-type (DNA) are intimately involved in the reproduction of linearly arranged gene proteins in the chromosomes whereas in the formation of cytoplasmic proteins and in the reproduction of simple viruses nucleic acids of pentose-type dominate. He contends that the nucleolus-associated chromatin which is rich in PNA exerts a controlling influence over nucleic acid metabolism generally, that materials which are formed in or about the nucleolus stimulate the appearance of heavily absorbing zones in the cytoplasm adjacent to the nuclear membrane, which zones are active centers for further elaboration of cytoplasmic protein. On the basis of a wealth of microabsorption data he maintains that the cell nucleus is the main but not the sole center for protein synthesis, also that the processes related to protein synthesis are of different nature in the nucleus and cytoplasm. He considers that the former serves as a central modulator of such mechanisms.

It should be emphasized that cytochemical studies which depend on differential stainability or ultraviolet absorptive capacity can quantitate only in the modes measured. If changes in the specific ultraviolet absorptive or dye-binding capacity of cellular constituents are to be interpreted as measures of changing concentrations or amounts of substances, then it must be first demonstrated, of course, that changes in the state of aggregation or composition of measured substances do not occur during the physiologic process under analysis. A recent study [63] has demonstrated that under certain circumstances calculated concentrations of cytoplasmic PNA as derived from ultraviolet microabsorption data may not, in fact, parallel PNA-P changes. Cytochemical data summarized in Table 3 indicate a decrease in the concentration of cytoplasmic PNA of liver cells during one hour of cold exposure. Bio-

TABLE 3. Mean Integrated Ultraviolet Extinction Values for Cytoplasm of Frozen-Dried Liver Cells and Supraoptic Neurons of Control Albino Rats and Litter-Mate Cold-Exposed Animals under Various Conditions. Percentage of Cytoplasmic Protein and Nucleic Acid Calculated from These Microabsorption Data

	Control										*One Hour Cold Exposure*									
	Liver					*Supraoptic Neurons*					*Liver*					*Supraoptic Neurons*				
Exper. Condition	#	E_{255}	E_{275}	*% Prot.*	*% N.A.*	#	E_{255}	E_{275}	*% Prot.*	*% N.A.*	#	E_{255}	E_{275}	*% Prot.*	*% N.A.*	#	E_{255}	E_{275}	*% Prot.*	*% N.A.*
Fed	5/13	0.30	0.26	^{1}22	1.8	5/19	0.26	0.20	9	2.1	4/10	0.26	0.29	31	1.5	3/6	0.25	0.25	23	1.6
		(.08)	(.08)				(.08)	(.07)				(.09)	(.09)				(.08)	(.08)		
				^{2}14	2.0															
				^{3}23	2.5															
				^{4}15	2.8															
				17	2.2															
18–24 hr. Fast	6/16	0.34	0.36	^{1}38	1.9	2/4	0.20	0.24	28	0.5	5/22	0.26	0.29	31	1.5	1/6	0.51	0.57	62	1.9
		(.11)	(.14)				(—)	(—)				(.16)	(.16)				(.03)	(.03)		
Bilat. Adrenalect. Fed + 1% NaCl	3/12	0.37	0.38	38	2.2	3/16	0.31	0.29	23	2.2	3/18	0.17	0.15	11	1.2	3/13	0.33	0.26	14	2.5
		(.11)	(.13)				(.18)	(.17)				(.11)	(.09)				(.13)	(.10)		

#: Number of animals / total number of integrated mean cytoplasmic values (approximately 75–150 separate values integrated in each).

E_{255} = Mean $E_{255} - E_{325}$ (5 μ frozen-dried section); E_{275} = Mean $E_{275} - E_{325}$ (5 μ section).

Subscript numbers in parentheses = standard deviation of mean.

% Prot. and % N.A. in grams $\times 10^{-14}$/cubic micron = grams protein or nucleic acid/100 ml. tissue.

Superscripts (1) (2) (3) (4) under Liver–% Prot. Control Fed, refer to values for four different sets of standard extinction values, viz., (1) N.A., $E_{255} = 0.125$, $E_{275} = 0.079$; Protein, $E_{255} = 0.0033$, $E_{275} = 0.0051$. (2) Same values for N.A.; Protein, $E_{255} = .0035$, $E_{275} = .007$. (3) N.A., $E_{255} = 0.88$, $E_{275} = 0.054$; Protein, $E_{255} = .0033$, $E_{275} = .0051$. (4) Same N.A. values as (3) and Protein, $E_{255} = .0035$, $E_{275} = .007$. All remaining % values calculated from following standard equations:

$$E_{255} = M_{N.A.} \cdot 0.107 + M.\,prot. \cdot 0.0034.$$
$$E_{275} = M_{N.A.} \cdot 0.067 + M.\,prot. \cdot 0.0061.$$

where $M_{N.A.}$ and M. prot. are grams $\times 10^{-14}/\mu^3$ of tissue for nucleic acid and protein respectively.

From Gordon and Nurnberger [63], Table I.

chemical analyses of the livers of comparable animals, recorded in Table 4, reveal an actual increase in concentration of PNA-P in homogenates of liver after brief cold exposure. A consistent elevation of PNA-P in all differentially sedimented cytoplasmic fractions analyzed is evident. Studies intended to explain this paradox have shown that brief cold-exposure substantially alters the sedimentation properties of the microsomal elements and, more importantly [64], that nucleotide ratios of purified liver PNA's from control and cold-exposed animals differ significantly, the latter showing a marked decrease in the relative molar complement of adenylic and uridylic nucleotides. Evidence for the fact [8, 73] that the sedimentation characteristics of PNA-rich cytoplasmic material is substantially different in young and relatively undifferentiated cells as contrasted with adult representatives has already been mentioned. These changes might be expected to reflect significant fluctuations in the mass or size of such particles. In this regard Caspersson demonstrated many years ago the critical dependence of over-all ultraviolet "absorptive" capacity on particle size [16]. Hence fluctuations in particle size may be partially responsible for the varying ultraviolet absorptive capacity of primitive as against adult tissues reported by Thorell and others, as also for some of the changes indicated in Table 3. Not only the microsomal particles of liver but also those of cerebral cortex showed substantially altered sedimentation characteristics following brief cold-exposure.

A shift in the degree of aggregation and orientation of cellular PNA has also been submitted [30] as a complicating factor in interpreting and quantifying ultraviolet microabsorption data. There is no question that highly aggregated PNA's absorb substantially less ultraviolet light per unit dry weight or mole phosphorus than do low molecular weight preparations [63, 66, 80, 93]. Whether such fluctuations in *in vivo* materials would be sufficient to influence microabsorption data is not clear. A few published studies [101, 136] cast doubt on the importance of this factor. No information is yet available on the possible effects of alterations in nucleotide composition on ultraviolet absorption or dye-binding capacity of PNA's. The majority of investigators who use dye-binding capacities as a measure of PNA concentration or content find fairly consistent gross correspondence with correlative chemical analyses. An occasional isolated study [52] suggests, however, that cytoplasmic basophilia does not necessarily give a true indication of the actual PNA concentration of certain mammalian cells under pathological conditions.

TABLE 4. A Typical Differential Centrifugal Fractionation of Sucrose Homogenates of Liver and Cerebral Cortex in Fed Control and 1 Hour Cold-Exposed Litter-Mate Wistar Rats

Tissue Homog. and Experimental Condition	*W.H.*			*N.F.*			*Mito. F.*			*Mic. F.*			*S.F.*			*% Recovery*		
	N. Gm. %	*DNA-P Mg. %*	*RNA-P Mg. %*	*N. Gm. %*	*DNA-P Mg. %*	*RNA-P Mg. %*	*N. Gm. %*	*N. % Tot.*	*RNA-P Mg.%*	*N. Gm. %*	*N. % Tot.*	*RNA-P Mg.%*	*N. Gm. %*	*N. % Tot.*	*RNA-P Mg. %*	*Tot. N.*	*Tot. RNA-P*	*Tot. DNA-P*
Liver Control Fed (2 animals)	2.64	23.3	102.7	.413	22.3	5.6	.578	21.9	14.8	.537	20.4	54.2	1.08	40.9	20.9	98.8	93.3	95.8
	(2.62	(22.8	(102.7	(.407	(21.8	(5.5	(.571		(13.9	(.537		(51.9	(1.06		(20.6			
	-2.66)	-23.7)	-102.7)	-.420)	-22.8)	-5.7)	-.585)		-15.7)	-.537)		-56.5)	-1.10)		-21.2)			
Liver-Fed 1 Hour Cold (2 animals)	3.07	22.3	111.0	.415	22.4	4.42	.595	19.3	15.9	.700	22.8	57.8	1.35	44.0	29.2	99.8	97.5	100
	(3.05	(21.8	(110	(.388	(22.2	(4.37	(.572		(15.1	(.630		(57.4	(1.31		(26.9			
	-3.10)	-22.8)	-112)	-.441)	-22.5)	-4.46)	-.598)		-16.6)	-.769)		-58.1)	-1.38)		-31.4)			
Brain Cortex Control Fed (2 animals pooled)	1.73	2.56	31.2	.097	2.40	3.63	.356	21.0	6.85	6.58	38.0	12.9	.574	33.0	13.4	97.5	118.0	93.8
Brain Cortex 1 Hour Cold (2 animals pooled)	1.59	2.85	32.8	.100	2.52	3.31	.347	20.8	6.45	.618	39.0	15.0	.541	34.0	13.3	99.0	116.0	88.5

W.H.: Whole homogenate (0.25 M sucrose).

N.F.: Nuclear fraction (600 g. for 10′ in 0.25 M. sucrose, 2 additional washes in 0.88 M. sucrose with resedimentations at 600 g. for 10′).

Mito. F.: Mitochondrial fraction (8500 g. for 10′ in 0.25 M. sucrose, 2 additional washes in 0.88 M. sucrose with resedimentations at 11,000 g. for 30′).

Mic. F.: Microsomal fraction (19,000–23,000 g. for 1 hour in 0.125 M. sucrose in isotonic saline, 2 runs).

S.F.: Supernatant fraction.

Numbers in parentheses indicate individual means of duplicate aliquots.

All values expressed as grams or milligrams per 100 grams of original wet weight of tissue.

From Gordon and Nurnberger [63], Table II.

The cells of the ventral hypothalamus provide a very favorable system for studying not only specific protein secretory substances but also the highly differentiated PNA-rich cytoplasmic structures previously alluded to as ergastoplasm. Scharrer and his co-workers [122] investigated secretory cycles in these cells and observed the progressive dissolution or disappearance of stainable ergastoplasm, more specifically Nissl substance, during accumulation of secretory granules in these cells. These observations are, of course, subject to the serious interpretive limitations already alluded to. Nonetheless this system promises to be a particularly favorable material for studying both cytochemical and ultrastructural correlates of metabolic activity, just as pancreas and parotid gland are.

CHEMICAL STUDIES OF PNA METABOLISM. A number of particularly informative studies have recently appeared on the possible role of PNA's or their constituent nucleotides and bases in bacterial metabolism. Reference has already been made to Bernheimer's [5] earlier observations on the relatively specific role played by certain PNA's in inducing or augmenting streptolysin formation in streptococci. Pardee [107] has recently demonstrated that pyrimidine-demanding mutants of *E. coli* require these compounds for induction of enzyme formation (see Table 5). He has shown that within minutes after the removal of pyrimidines from the medium of such mutants induction of enzyme ceases. His own conclusions are that the PNA's found in such bacterial cells are largely inert metabolic products and that the continuous formation of new PNA is essential for the protein synthesis implicit in such enzyme induction. These results are not entirely clear cut because he was dealing with pyrimidine-demanding mutants. The evidence of Gale and Folkes [58] is more definitive. They demonstrated not only that the exchange rate of labeled glutamic acid with proteins in disrupted staphylococcus aureus preparations was greatly augmented by the addition of staphylococcal PNA (see Text Fig. F) but also that the level of catalase and beta galactosidase activity in such preparations freed of intrinsic PNA was greatly augmented by the reintroduction of PNA or a purine-pyrimidine mixture (Text Fig. G). Increases in protein nitrogen up to 31 per cent were observed in the latter preparations. These workers concluded, too, that it is primarily newly formed PNA which functions in such protein synthesizing processes, and their evidence is clearly spelled out in that work. These studies leave little doubt that the metabolic activities of PNA's are somehow intimately involved with processes resulting in new protein formation. The mechanisms of action of PNA or its constituent nucleotides are of course only hinted at in these studies. Hypotheses mentioned lean heavily on the presumed organizing potential reflected in structural specificities of the PNA molecule as mentioned years before both for PNA [20] and for the DNA transforming factor of bacteria [21]. The question of whether mononucleotides now shown to exist in various tissue extracts can serve as precursors for new and active PNA during cellular protein synthesis is not answered by these experiments. It is entirely possible that preformed cellular PNA may serve as a labile source in living mammalian cells for mononucleotide precursors of new PNA's required for specific protein synthesis. Observations already mentioned [64] are consistent with such a possibility.

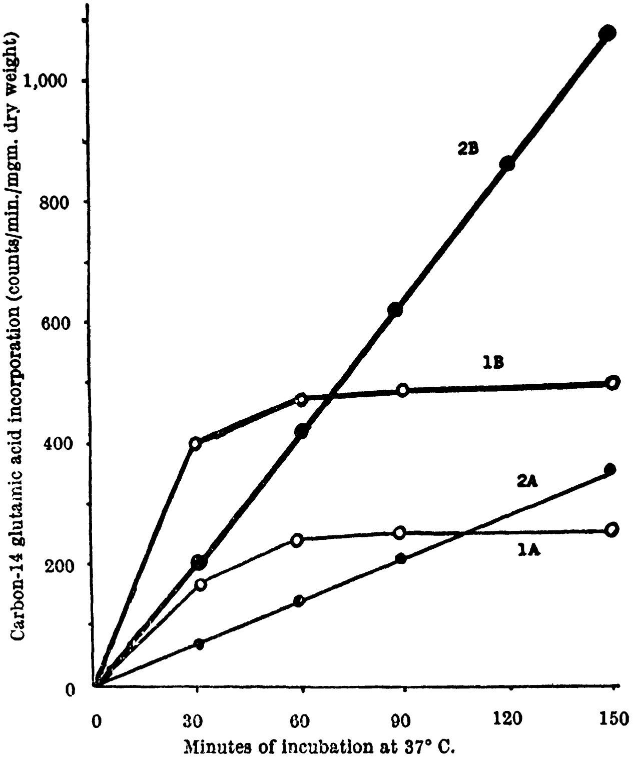
TABLE 5. Percentage Changes in Protein and Nucleic Acids after Induction in Pyrimidine-Requiring Mutants of *E. coli*

Time (Min.)	*Protein*	*DNA*	*RNA*
		Mutant 550–460	
No pyrimidine:			
3	100	100	100
10	98	94	93
20	102	89	90
30	94	120	103
50	113	105	92
80	100	76	96
Excess uracil:			
30	134	(190)	145
80	213	272	276
		Mutant 6386	
No pyrimidine:			
0	100	100	100
4	100	104	112
12	108	98	102
25	110	101	111
50	120	106	110
Excess uracil:			
25	123	137	155
50	165	161	214

Figures represent percentage changes over the initial sample in the presence or absence of uracil. From Pardee [107], Table III.

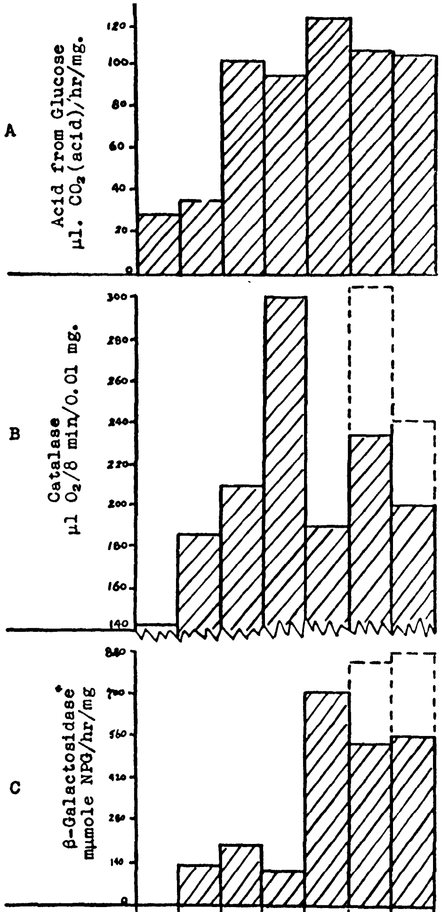
Davidson, Leslie, and associates [35, 36, 88] have measured the rates of accumulation of protein nitrogen, lipide phosphorus, and the phosphorus of RNA in growing organs and tissue cultures after Schmidt-

Thannhauser separation of RNA from DNA. They have referred all of these to the DNA phosphorus of the organs and tissues studied on the assumption that DNA phosphorus bears a relatively constant relationship to cell number. Text Figure H summarizes some of their relevant data. In this figure the slope of the protein nitrogen and RNA-P accumulation curves for embryonic chick heart are very nearly identical. This fact suggests that the rates of cellular accumulation (or synthesis) of



TEXT FIGURE F. Course of incorporation of ^{14}C-labeled glutamic acid by disrupted cell preparation. Curve 1A: preparation incubated with adenosine triphosphate, hexose-diphosphate and alpha-carboxyl-^{14}C-glutamic acid. Curve 1B: as for 1A with addition of 0.1 mgm staphylococcal nucleic acid/ml. Curve 2A: as for 1A with addition of complete mixture of amino acids. Curve 2B: as for 1B with addition of complete mixture of amino acids. Counts carried out on protein fraction of preparation in all cases. From Gale and Folkes [58].

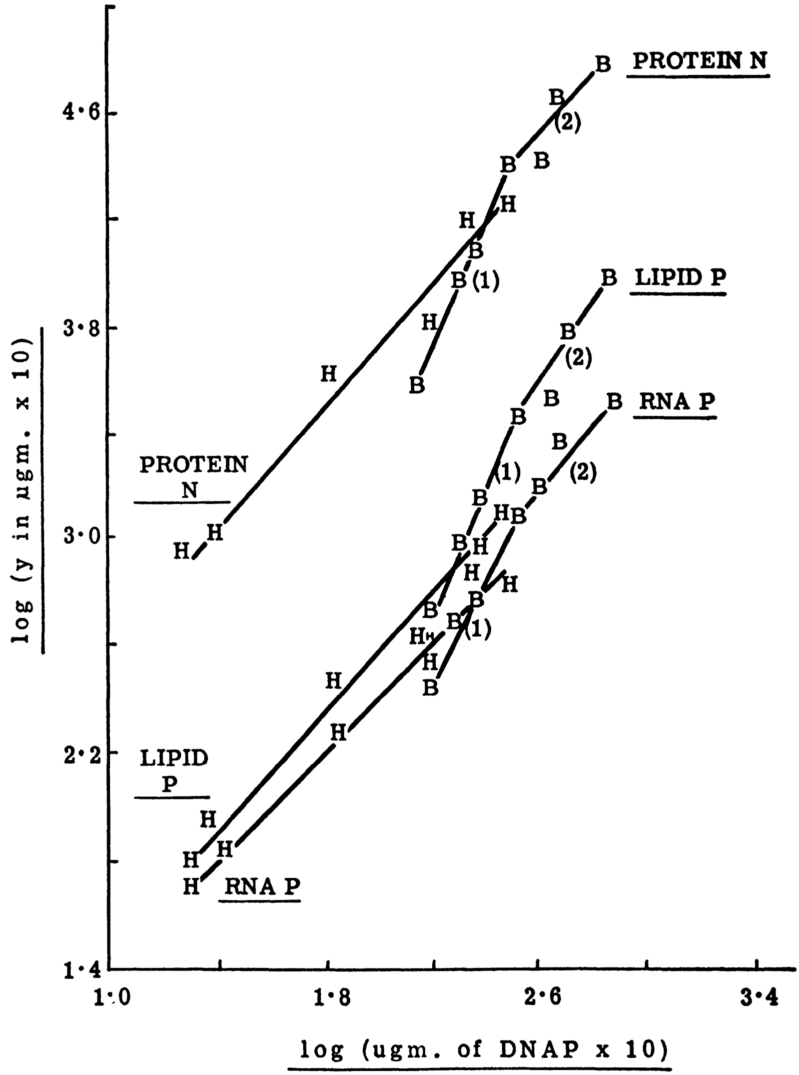
these metabolites run closely parallel. A similar relationship is noted for the rate of accumulation per cell of protein nitrogen and RNA-P in chick brain. This figure reveals that lipide phosphorus also accumulates in brain and at a rate greater than either RNA-P or protein nitrogen. Roughly comparable trends have been observed by Flexner and Flexner [54, 55] in developing fetal guinea pig brain and liver. Such studies, however, which detail simultaneous rates of accumulation



ATP,HDP	+	+	+	+	+	+	+
Aminoacids		+	+	+	+	+	+
DNA			+			+	
RNA				+			+
Pur./Pyrim.					+	+	+

TEXT FIGURE G. Development of enzyme system forming acid from (A) glucose, (B) catalase, and (C) betagalactosidase in disrupted cell preparations. Time of incubation: A, 90 min.; B, 90 min.; C, 120 min. *, 2 per cent galactose present in incubation mixture for experiments on betagalactosidase development. Broken lines represent occasional atypical results. From Gale and Folkes [58]. Conditions of incubation and quantities used indicated in that reference.

or disappearance of protein nitrogen and PNA-P provide only inferential evidence for the possible metabolic interrelationship of protein synthesis, cell growth, and PNA. The physiological literature is supercharged with paradoxical data which could be interpreted *ad absurdum* along the



TEXT FIGURE H. Growth ratios of protein nitrogen, lipide phosphorus, and ribonucleic acid phosphorus relative to deoxyribonucleic acid phosphorus content of embryonic chick heart (H) and brain (B). From Davidson and Leslie [35], Fig. 4.

same lines. To mention just one generally accepted example, pituitary gonadotropins appear in extraordinarily high titre in the blood of castrate or involutional females, the titres, in fact, being far higher than those observed under normal physiologic circumstances during which the end-organs are actively metabolizing. Nonetheless, no student would be tempted to infer that the elevated gonadotropin titre is a cause of gonadal involution even though a relationship, albeit inverse, does exist. Metabolites often enough increase in amount or concentration in cells or body fluids because they are not being utilized, excreted, or broken down. The pitfalls awaiting those who are tempted to interpret concentration shifts or accumulation data are real even though the resultant absurdities may be more carefully hidden than in the example cited.

Elson and Chargaff [50] have estimated the total PNA of growing sea urchin embryos during the first forty-eight hours following fertilization in terms of the sum of constituent nucleotides analyzed by chromotographic techniques. They discovered that the PNA remained fairly constant in amount as well as in nucleotide composition during the period studied, while at the same time DNA rose from extremely small absolute amounts to approximately 2½ per cent of dry weight. This study makes improbable the conversion of PNA to DNA, as suggested by previous workers. If the DNA in this system is even an approximate measure of total cell numbers, it would appear that the PNA content per cell in the growing sea urchin embryo decreases quite rapidly during this particular phase of rapid development.

Chemical studies on rapidly regenerating and protein synthesizing adult organs have generally been involved with radio-isotope uptake and turnover rates. Several well-controlled biochemical experiments will be described before such labeling experiments are considered. The results of a carefully quantitated analysis of liver regeneration by Drabkin and his associates [44] are detailed in Table 6 from his published results. The dietary conditions established in this study were quite stringent, all animals having been maintained for thirteen days preoperatively on no protein or high protein intake and continued for thirteen days postoperatively on the same regime with twenty-four-hour fasts before operation and sacrifice. The noted extraordinary increase in the concentration of PNA in regenerating liver on protein-free diets obscures the fact that the actual total amount of PNA in regenerating liver under the two dietary regimes is not significantly different. This is calculated to be on the average 40.7 milligrams per liver on a protein-free diet and 38.6

TABLE 6. Ribose Nucleic Acid (PNA) and Desoxyribose Nucleic Acid (DNA) after 14 Days Restoration, Following Excision of 68.4 Per Cent of Liver

Rat No. (*1*)	*Liver Excised* (2)		*Weight of Original Total Liver* (3)	*Remaining Liver* (4)			*Final Total Liver* (5)		*Nucleic Acid in Final Liver* (6)		*New or Restored Liver Tissue* (7)	*New Nucleic Acid in Restored Liver* † (*8*)			
	Amount	W:D *		*Amount*	PNA	DNA	*Amount*	W:D	PNA	DNA		PNA		DNA	
	gm.		*gm.*	*gm.*	*mg. per gm.*‡	*mg. per gm.*‡	*gm.*		*mg. per gm.*‡	*mg. per gm.*‡	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
64	3.75	3.89	5.48	1.73	9.60	2.36	3.66	3.83	13.10	3.09	51.4	31.3	65.3	7.2	63.7
65	2.78	3.92	4.07	1.29	10.20	2.73	2.85	3.70	12.50	3.06	56.1	22.4	62.9	5.2	59.8
66	5.21	3.89	7.62	2.41	9.45	2.58									
67	3.29	3.71	4.82	1.53	9.80	2.75	3.45	3.92	11.20	3.01	58.4	23.6	61.2	6.2	59.6
68	4.43	3.21	6.48	2.05	8.10	2.44	5.65	3.51	6.80	3.52	81.3	21.8	56.7	14.9	74.8
69	4.37	3.35	6.39	2.02	8.10	2.50	5.22	3.40	7.55	3.43	73.2	23.0	58.3	12.8	71.5
70	4.93	3.35	7.21	2.28	9.10	2.47	5.28	3.28	7.10	3.58	60.8	16.7	44.5	13.3	70.3
71	5.03	3.39	7.35	2.32	8.70	2.41	6.15	3.17	6.40	3.58	76.1	19.2	48.7	16.4	74.5
Mean ± s.e.		3.85			9.76	2.60		3.82	12.27	3.05	55.3	25.8	63.1	6.2	61.0
(N. P.) §		±0.05			±0.16	±0.09		±0.06	±0.55	±0.03					
Mean ± s.e.		3.32			8.50	2.46		3.34	6.96	3.53	72.9	20.2	52.1	14.4	72.8
(H. P.) ||		±0.04			±0.25	±0.02		±0.07	±0.24	±0.04					

The analyses of PNA and DNA were performed in each case on duplicate aliquots of tissue.

* W:D represents ratio of Wet Weight (W) to Dry Weight (D).

‡ Wet weight of tissue.

§ N.P. = protein-free diet, Rats 64 to 67.

|| H.P. = high protein diet, Rats 68 to 71.

From Drabkin and associates [44], Table V († calculations for new nucleic acid formed are described in that reference).

milligrams per liver on a high-protein diet. There is, however, a significant difference in the accumulation of PNA and DNA under these different dietary conditions. Thus the PNA to DNA ratio in remaining and final livers is 3.9 and 4.0 respectively on the protein-free diet and 3.46 and 1.96 on the high-protein diet. This study has been interpreted to indicate that cytoplasmic PNA is preferentially synthesized during liver regeneration when dietary protein is severely restricted. Of particular interest was the observation not noted in this table, that unusually great liver regeneration followed the introduction of dietary protein supplements after a period of regeneration on protein-free diet. It is quite tempting to draw an analogy between this particular observation and that of Gale and Folkes (see Text Fig. F) on the markedly augmented incorporation of labeled glutamic acid by disintegrated PNA-poor *Staphyloccus aureus* preparations after the addition of supplemental complete amino acid mixtures and PNA. As regards the synthesis of specific protein enzymes during liver regeneration, Daoust and associates [33] found no significant changes in the amounts of either DNA-ase or RNA-ase per cell during the regenerative process. Hence the synthesis of these particular enzymes appeared to keep pace with cell division during regeneration.

The pancreas has proved to be a particularly revealing organ for a study of the metabolic potentialities of PNA not only because it is extraordinarily rich in this nucleic acid and its corresponding ribonucleases, but because it is an extremely active synthesizer of protein substances and therefore serves as a pilot organ for investigation of interrelationships between protein synthesis and PNA. Recently published observations indicate that the PNA content of pancreas remains relatively constant in various phases of its secretory cycle even after stimulation of protein synthesis by the addition of an amino acid mixture [75] or injection of pilocarpine [112]. PNA was estimated in these experiments as PNA-P. Hokin and Hokin [75], in discussion of their data, express the generalization that in dividing embryonic cells the PNA content and rate of protein synthesis are found to parallel one another, as exemplified in Text Figure H, because under these circumstances all the essential components of the cell are reproduced at a rate consistent with the formation of these substances at physiologic levels in the resultant new cells. Such relations no longer obtain in protein-synthesizing but nondividing mature cells, according to them. Chemical analyses of pancreatic activity may, if they are based solely on PNA-P, ail to reveal pancreatic cellular phenomena reported by Caspersson 17, 18], since his observations were of ultraviolet absorptive capacity.

In contrast to the already described studies of Hydén and his associates on absorptive alterations in functioning neural tissue, Ehrlich and Dische [48] could detect no change in the PNA content of mammalian retina following light stimulation. The mammalian retina is, of course, a highly complex organ in which neurons represent a very small proportion of the total cellular complement. In addition to this, the modes of measurement are again significantly different in the two studies; hence a direct comparison of such data is entirely unjustified. For a comprehensive survey of published chemical studies based almost exclusively on tissue phosphorus analyses, reference should be made to Leslie's recent review [87]. His summary concludes that changes in DNA content of a tissue or organ always appear to be linked with changes in cell number, whereas the amount of PNA depends on the structure's physiological function and state. The general implication, however, that a change in the amount of RNA-P or degree of specific ultraviolet absorptive capacity in a tissue preparation need necessarily reflect a change in amount of PNA or, perhaps more importantly, that the metabolic activities of a PNA must necessarily be reflected in over-all changes in its concentration or amount, is not clearly established. Even for DNA, qualitative fluctuations which may not alter over-all phosphorus content or ultraviolet absorptive capacity must be entertained as possible. As an example, labile shifts in the ratios of constituent nucleotides reflecting very great activity might not necessarily be associated with any change in the phosphorus or ultraviolet absorptive capacity of a nucleic acid isolated from such functioning tissues. An additional complicating factor in the interpretation of data reviewed here and by Leslie is indicated by Drasher [45], who has reported that acid precipitation after alkaline hydrolysis of mixtures containing both DNA and PNA does not always effectively separate the DNA. Some DNA may still appear in the acid-soluble supernate. This difficulty would apply only to data obtained by the Schmidt-Thannhauser technique. Major problems in the interpretation of chemical data for neural tissue have recently been summarized by Logan and associates [89].

THE METABOLIC ACTIVITIES OF PNA'S AS REVEALED BY ISOTOPE LABELING STUDIES. A variety of studies have followed the rate of exchange and incorporation of isotopic nitrogen, carbon, and phosphorus into the PNA's of physiologically active organs. Additional information of great interest has accrued from analysis of the relative rates of incorporation of such label into the particulate and nonparticulate fractions of tissue homogenates and more recently into the individual nucleotides of cellular fractions under many different circumstances. Finally, a comprehensive investigation of the likely and presumed precursors of cellular nucleic acids has been carried on by the same revealing techniques. A brief survey of these results is given here. Comprehensive reviews such as those of Brown and Roll [13] and Smellie [127] are available for exhaustive documentation.

Neither DeDeken-Grenson [39] nor Hokin [74] could find any significant change in the relatively slow renewal rate of PNA-P of pancreas after procedures known to stimulate the rapid synthesis of proteins. DeDeken-Grenson [38] has reported no relationship between the rate of protein synthesis in the oviduct of hen and the rate of renewal of PNA-P in that same organ. This particular interpretation may be questioned unless, as he implies, the rate of protein synthesis and PNA-P phosphorus renewal are related only when a 1:1 quantitative relationship obtains. Hokin did demonstrate [74] that the stimulation of pancreatic secretions by carbamylcholine produces a substantial increase in rate of labeled phosphorus uptake by pancreatic PNA. On this basis he suggested that PNA may function in rearranging and perhaps mobilizing proteins during the secretory process. Koritz and Chantrenne [85] have followed the maturation of reticulocytes to mature red blood cells in phenylhydrazine-treated rabbits. They observed that the incorporation of labeled carboxyl carbon from glycine into reticulocyte proteins increases in approximately linear fashion for four hours, the peak incorporation occurring one to two days before the PNA content of these cells reaches its maximum. The production of carbonic anhydrase, peptidase, and hemoglobin approached a maximum at about the same time as did PNA. They concluded that the PNA content in itself is not a limiting factor for the incorporation of amino acids into reticulocyte protein though PNA may in fact be involved in the process of protein differentiation. Such a role of PNA would, in their opinion, require many PNA's for providing stereo- and other specificities. The relationships reported in this study are at variance with cytochemical data of Thorell (see Text Fig. D) already alluded to. Koritz and Chantrenne's observations are, however, in accord with those of Gale and Folkes, Pardee, and Bernheimer, which establish a definite relationship between the presence of PNA or new formation of PNA and the elaboration of such specific proteins as streptolysin, catalase, and beta galactosidase.

Pioneer investigations [34, 72] indicating a far more rapid turnover of PNA-P and PNA-N in resting adult liver than the corresponding elements in DNA have generally been borne out by subsequent studies. It has also been confirmed that the turnover of label in DNA far exceeds resting adult rates in cells undergoing rapid division as during embryogenesis, or liver regeneration [34, 125]. The time schedule for the incorporation of labeled nitrogen from glycine was studied in regenerating rat liver PNA by Eliasson, Hammarsten, and associates [49] some years ago. They noted that the maximum turnover rate for the nitrogen particularly of the purine bases of PNA nucleotides reached a maximum at about the thirtieth hour following partial hepatectomy, the maximum rate of nitrogen incorporation into the amino acids of proteins occurring around the sixtieth postoperative hour. Here again the maximal activity of the polynucleotides preceded maximal nitrogen incorporation into the amino acids of liver proteins. In regenerating nerve, where an intrinsically slow renewal of axoplasm but a relatively rapid multiplication of Schwann cells is observed, a sharp increase in the exchange rate of pentosenucleoprotein [121] and an increase in the PNA to DNA ratio during the first two weeks following section or crush [89] have been found. Nervous tissue is a particularly trying one in which to attempt either nucleic acid fractionations or turnover estimates. Deluca, Rossiter, and Strickland [41] have shown that more than one-half of protein-bound phosphorus in gray matter of brain (which appears in Schmidt-Thannhauser hydrolysates as organic phosphorus) does not absorb as do nucleotides in the ultraviolet. In white matter this non-nucleotide, protein-bound phosphorus of the same hydrolysates may account for as much as 80 per cent of the phosphorus of the PNA fraction. Moreover, it has a far greater specific activity than PNA phosphorus itself. A chromogenic material in neural tissues which interferes with the color reactions for phosphorus in the Schneider technique [89] has also been described. Studies particularly on the turnover of labeled phosphate in liver are, as Davidson and Smellie [37] have shown, seriously complicated by the presence of highly active non-PNA phosphorus in Schmidt-Thannhauser fractions. They find that all of the phosphorus containing components of the nuclei of liver have higher specific activities than the nucleotides two hours after administration of ^{32}P. Of six non-nucleotide fractions isolated by them, one absorbed ultraviolet and five of the six were organic phosphorus-containing fractions. They estimated that approximately 75 per cent of total phosphorus in the so-called ribonucleotide fraction obtained by the Schmidt-Thannhauser method is RNA-P, the remaining 25 per cent being non-nucleotide phosphorus which generally turns over at a rate far more rapid than that of the nucleotides. Before we turn to studies of specific turnover rates in isolated cell fractions and in component mononucleotides, a recent study by Findlay, Rossiter, and Strickland [53] should be mentioned. They noted a quite significant dependence of phosphate incorporation into brain PNA on oxidative mechanisms. Phosphorus incorporation was greatly decreased by creating anaerobic conditions, dispersing the tissues, or by adding a wide range of agents that depress oxygen consumption. They suggested a parallel between conditions which reduce or prevent the incorporation of labeled phosphate into brain PNA and those which cause a failure of respiring brain slices to maintain usual concentrations of phosphocreatine. In kidney extracts Pavlova [109] has suggested that oxidative phosphorylation of PNA involves only PNA less firmly bound to protein. Ribonuclease sharply repressed respiration and phosphorylation of PNA in these extracts but not after removal of proteolytic enzyme contaminants.

Bergstrand [4] and his associates reported in 1949 that guanine and adenine from the PNA's of cell nuclei in regenerating liver incorporated more nitrogen from labeled glycine than did these same bases from liver cytoplasm. In this early study they also observed that uridine from normal cytoplasm had a higher activity than guanine and adenine. The latter base had a higher turnover rate than either uridine or guanine in regenerating cytoplasm. These data are somewhat difficult to interpret in the light of present chromotographic information. The authors mentioned, for example, that the uridine analyzed derived in part from cytidine in their pyridine hydrolysates. In 1950, Marshak and Vogel [98] reported a greater turnover of labeled phosphorus in nuclear PNA than cytoplasmic PNA of rat and rabbit liver and indicated that at least 90 per cent of the label in the nuclear fractions was in nucleotides. Their observation that at any stated time after administration of label the specific activity of the various nucleotides separated were all approximately the same except for that of the guanylic, which was low, is not in complete agreement with more recent data [126]. Investigations [99, 128, 146] have subsequently confirmed that liver nuclear PNA incorporates labeled phosphorus and nitrogen more rapidly than any cytoplasmic fraction. Smellie has pointed up the important consideration [127] that although nuclear PNA is a relatively insignificant component of the entire cell, it exhibits such relatively prompt and high metabolic activity that the total turnover of nuclear PNA in a given time, more especially within the first four to six hours, is not very much smaller than the total turnover of cytoplasmic PNA during this time. In contrast with PNA activities, cytoplasmic protein fractions incorporate labeled phenylalanine more rapidly than do isolated and partially purified proteins of the nucleus [14].

In several recent studies [82, 128, 146] the turnover of phosphorus in the nonparticulate supernate of liver cytoplasmic PNA is found to proceed at a more rapid rate than that of any particulate cytoplasmic component. In contrast, protein of the microsomal fraction of rat liver homogenates incorporates label, for example from alanine [126], more rapidly than any other cytoplasmic component. Khesin [82] states that the order of decreasing activities for PNA-P of normal liver cytoplasm is cell sap, microsomes, mitochondria; but he reports that the turnover rate of labeled phosphorus in mitochondria is increased rapidly during regeneration and also during refeeding after starvation. He reports a more rapid turnover of RNA-P in mitochondria than in microsomes of the mammary glands of lactating rats, also in isolated microchondrial fractions from whole embryos during active growth. Because of known rapid alterations in the sedimentation characteristics of microsomes during physiologic activity, more especially in view of the fact that in embryonic tissue relatively large amounts of cytoplasmic PNA are not sedimented even at extremely high gravitational forces [8], it would seem more revealing to compare such reported mitochondrial turnover rates with those of the total supernates after mitochondrial sedimentation. Information for such comparative calculations from Khesin's experiments is unfortunately not available. However, Smellie has summarized data [127] indicating that in the mouse embryo incorporation of labeled phosphorus by nonsedimented PNA is the greatest, mitochondrial activity being next, that of the microsomes least. This certainly suggests that the distinctions drawn by Khesin for the embryo are more a reflection of altered sedimentation characteristics of fractions than a significant change in the incorporating activity of mitochondrial PNA's with stage of development.

Smellie and associates [128] have reported that in all cellular fractions, labeled carbon is most rapidly incorporated into the adenylic nucleotides of PNA. In whole cytoplasm the rate of incorporation of labeled carbon into the constituent nucleotides of PNA is in the following decreasing order: adenylic, guanylic, cytidylic, uridylic. Nuclear PNA, in contrast, shows the greatest incorporation rates for adenylic and cytidylic. Deluca and associates [41] report somewhat greater activities for adenylic and uridylic nucleotides in the PNA of cat brain slices which are respiring in Krebs-Ringer bicarbonate medium containing glucose and labeled phosphate. This observation is more in line with the conclusions recently drawn by Smellie [127] in his critical survey of recently published data. Here he states that the activity of the four pentose mononucleotides is approximately in inverse proportion to their relative molar proportions within PNA. We would, therefore, expect that the adenylic and uridylic nucleotides of both brain and liver PNA would incorporate label more rapidly than those of cytidylic or guanylic nucleotides. In spite of some inconsistencies in labeling experiments, the relative activities of uridylic and adenylic nucleotides, particularly in rat liver, generally do exceed those of the remaining two nucleotides [4, 7, 128].

Caspersson and his co-workers have placed great emphasis on the specific role of the nucleus in protein metabolism based on cytochemically estimated concentration shifts during cellular activity, protein synthesis, cell division, and microbiological growth. In this light recent observations concerning the significantly different activities of nuclear and cytoplasmic PNA's are of particular interest. Smellie [127] has critically evaluated published data on time-activity curves for turnover of nuclear and cytoplasmic PNA's which support the contention that there is a common precursor for both nuclear and nonparticulate cytoplasmic PNA, the latter serving as a source or reservoir for particulate cytoplasmic PNA. These time-activity relations are not, in Smellie's opinion, consistent with the hypothesis that nuclear PNA can serve as precursor for cytoplasmic PNA. Brachet has published data [9] on the incorporation of labeled orotic acid into the PNA's of *Acetabularia mediterrania,* which imply a constant dissociation between the activity of nuclear and cytoplasmic PNA, even though the nucleus of this unicellular organism appears to be necessary for the prolonged maintenance of microsomal integrity.

In spite of the fact that an intimate and even specific relationship between PNA activity and protein synthesis has long been presumed, relatively few direct tests of this hypothesis have been attempted, except for the microbiological experiments of Pardee, Gale and Folkes, and

Bernheimer already alluded to. Biological systems which permit a critical analysis of these interrelationships are now available. In 1952 Siekevitz [126] reported that the proteins of microsomal fractions of rat liver homogenates showed the greatest incorporation of labeled alanine of any separated fraction. Either mitochondria or a soluble fraction obtainable from disintegrated mitochondria was required to support such incorporation. Alpha ketoglutaric acid, various cofactors required for its oxidation, and finally aerobic conditions all increased the uptake of alanine into this fraction from five- to ninefold. It was his conclusion that concomitant phosphorylative mechanisms, and not precisely oxidation of substrate as such, were responsible for such amino acid incorporation. Following this lead Allfrey, Daly, and Mirsky [1] pre-incubated rat liver microsomal fractions with crystalline ribonuclease. Appropriate controls were incubated in the substrate without enzyme. After fifteen minutes of enzymatic or substrate incubation fresh sucrose suspensions of mitochondria were added and the mixture incubated with DL-alanine-1-^{14}C. The specific activity of the ribonuclease-treated microsomal protein was approximately one-half of that in control preparations. They also observed that no appreciable incorporation of amino acid occurred in the absence of oxidizable substrate. It is apparent from this crucial study that a highly active mammalian system has been defined which can well serve as an experimental model for the critical investigations of specific interrelationships between PNA activities and amino acid incorporation. With such a system it should soon be possible to test the hypothesis of Pardee, and Gale and Folkes that only newly synthesized PNA is effective in supporting protein synthesis. Through a study of specific protein moieties elaborated in the presence of structurally defined PNA's it should also be possible to test whether or not a given macromolecular PNA can impose certain structural specificities on new protein. Before this can be accomplished, however, more definitive information on the sequence of individual nucleotides in PNA's must be obtained. The analytical problems are indeed formidable but the tools and model systems are now at hand.

THE BIOSYNTHESIS OF PNA'S AND THEIR PRESUMED PHYSIOLOGIC PRECURSORS. The hypothesis that PNA's may serve as highly structured yet exquisitely labile cellular sources of high energy phosphate compounds or their precursors has enjoyed considerable popularity for almost ten years. Studies on the fundamental structure of PNA's, already summarized, are compatible with the proposal that nucleoside-5-phosphates (which can be readily liberated from macromolecular PNA by known enzymatic mechanisms) are in a dynamic state of equilibrium with nucleotide di- and triphosphates recently demonstrated in a variety of tissues. Since biosynthetic pathways for PNA would very likely be involved in such an equilibrium, long-term studies of Brown and Roll [13] on the biosynthesis of PNA's must be mentioned here. Summarizing their own work and that of many others in the field, they have recently stated that both purine and pyrimidine nucleotides have been found in every case except that of the adenylic acids to be utilized as readily as, or more extensively than, any of the simpler derivatives in the biosynthesis of PNA's. They direct particular attention to the guanylic acids, since, at least in the rat, their purine moieties are utilized far more readily than are any of the simpler derivatives. They urge caution, however, in drawing any conclusions concerning potential relationships of currently recognized metabolites in the acid-soluble fractions of tissues to the actual or supposed "active" polynucleotide precursors. The specific reason for this caution is not noted. In their review they propose as a central purine precursor "active adenine," which may be converted into and be in equilibrium with the larger cellular reservoir of adenosine-5′-phosphates. They entertain serious reservations as to whether AMP is in active or rapid direct equilibrium with the presumed active precursor, since they have noted that administered AMP is approximately one-half as extensively incorporated into PNA purines as are equimolar amounts of the corresponding 2′ and 3′ isomeric nucleosides. The fundamental questions which they raise as to whether the active biosynthesis of polynucleotides in tissues proceeds by a stepwise build-up, unit by unit, or whether in fact there is a simultaneous assembling of all components, are currently as near solution as quite analogous problems concerning biosynthesis of proteins. In presumptive support of a stepwise synthesis or partial renewal, they mention the manifest chemical heterogeneities of PNA preparations and the unequal incorporation rates of purine and pyrimidine precursors into different units of any given nucleic acid. Thus available biosynthetic data are consistent with, but in no way establish, the activity of cytoplasmic PNA's as labile high energy mononucleotide reservoirs. In defense of this proposal it should be mentioned that exchange reactions between PNA and specific nucleotides may involve mechanisms of quite different order from those involved in synthesizing polynucleotide *de novo*. Dancis and Balis [32] have reported that labeled purine moieties which enter the blood stream as a result of the catabolism of tissue nucleic acids are not reutilized in the rat to any great extent for nucleic acid biosynthesis. This, however, cannot be interpreted as evidence against the utilization of known precursors occurring within the cells themselves. Again, both the model systems and the techniques are available for solving these particular problems in the immediate future.

The use of analogues of the purines or pyrimidines in studying alterations in nucleic acid metabolism has been rather restricted in general physiological studies, though such compounds have of course been extensively studied by Brown and his associates as potential precursors of PNA constituents. The conspicuous therapeutic effects of methylthiouracil in decreasing thyroid activity have been associated with alterations in the metabolic activities of both PNA and DNA in the thyroid itself. An attempt was made by Reřábek [114] to demonstrate that 4-methyl-2-thiouracil might, in fact, serve as a precursor of PNA in liver cells. His published observations, however, fail to establish any convincing relationship between the administration of this analogue and PNA metabolism. They certainly do not prove that it can be used for the biosynthesis of liver cytoplasmic nucleotides, since none of this analogue base was shown to have been incorporated into nucleotide. If such analogues could, however, be incorporated into PNA's in significant amounts, resultant alterations in stereo- and other specificities might be of value in elucidating PNA-protein synthetic interrelationships. Along this line, Jeener [78] has studied the influence of thiouracil incorporations into the PNA moiety of tobacco mosaic virus on its multiplication rate. Apparently insufficient thiouracil was incorporated to inhibit mutliplication of all elementary units of each virus particle, so that the resultant particles did remain infective. His data supported the contention that the proportion of elementary units capable of being duplicated in the host were, however, reduced. Further studies of such nature might well provide information of great importance concerning the presumed specific biological role of such materials in the reduplication or synthesis of proteins.

Microbiological systems are unusually favorable ones for studying the metabolic interrelationships of proteins and PNA and have already provided important information. Cohen [28] almost ten years ago observed that during the multiplication of T_2r bacteriophage in *E. coli* the DNA is actively synthesized from inorganic phosphate of the medium while the PNA of *E. coli* itself remains relatively inert. Even in these early studies he observed that nucleosides stimulated virus multiplication and were much more effective in this respect than such constituent bases as guanine.

4. Extrinsic Effects on PNA Metabolism

Many published studies on the effects of extrinsic agents and events on nucleic acid metabolism have contributed relatively little to our knowledge of the metabolic role of PNA's for several reasons. The techniques of isolation and analysis have frequently suffered from limitations which have already been mentioned. Very often the effects of contraction or expansion of cellular compartments, particularly as effected by dietary or hormonal regimes, have not been adequately appraised. In many experiments nonspecific factors such as stress or nutritional alterations attendant upon experimental regimes have not been dissected away from presumed specific effects.

HORMONAL FACTORS. Cytochemical and biochemical studies [63, 105] have revealed that there is a significant decrease in the specific ultraviolet absorptive capacity of the cytoplasm of hypothalamic neurons and of liver cells in the albino rat with brief cold-exposure. This type of environmental stress is one of many known to activate the hypothalamo-pituitary system and, in a matter of minutes, to stimulate the adrenal cortex to elaborate its specific corticoids in more than usual amounts. During cold-exposure substantial alterations in the magnitude of the cellular compartment of liver occur. These account reasonably well for some of the shifts in cytoplasmic protein concentration indicated by cytochemical studies. Chemical analyses, however, indicate an increase rather than a decrease in PNA-P, during the interval when selective cytoplasmic ultraviolet absorption is changing in reverse direction (see Tables 3 and 4). The microsomal fraction of liver as well as of cerebral cortex does not sediment completely after such brief stress. Subsequent studies already alluded to [64] suggest that significant changes in the ratios of constituent nucleotides in the cellular PNA's of these organs may provide a partial explanation for the significantly altered ultraviolet absorptive capacity of such cells.

Cytochemical studies in bilaterally adrenalectomized salt-maintained rats [63] indicate that the cytoplasmic ultraviolet absorptive capacity of autonomic neurons and liver cells is not significantly changed from control values except for a shift of maximal absorption toward longer wave lengths, suggesting increased protein absorption. In bilaterally adrenalectomized animals briefly exposed to cold, however, there are profound alterations in the ultraviolet absorptive capacity of the cytoplasm of both cell types, with a marked decrease in long-wave-length absorption in both. In liver cells a decrease in ultraviolet absorption at the nucleic acid maximum also occurs. These data are in general agreement with those of Smith [131]. He found that adrenalectomy itself does not appear to affect either PNA or DNA levels in the tissues of fed rats, but that there was a marked total decrease in PNA content in the liver of adrenalectomized rats following the stress of a twenty-four-hour fast. No significant changes were observed under the same conditions in kidney, whole brain, or skeletal muscle. Lowe and his associates have published data [92] indicating that cortisone produces a marked expansion of the cellular compartment in rabbit liver, though their observations were not interpreted in this way by them. In a subsequent study [91] they report that after cortisone treatment, microsomes and mitochondria are no longer completely sedimented from liver homogenates in contrast to controls. They also observe an increase in the rate of incorporation of ^{32}P into the soluble supernatant fraction of liver homogenates in cortisone-treated animals. They propose that the PNA of the soluble fraction is continuously converted to particulate PNA during normal cellular activity and that cortisone prevents this conversion. The changes described in these studies have far broader implications than appear at first glance. Subsidiary evidence indicates that any procedure which imposes an unaccustomed environmental stress on the organism might be expected to initiate the reactions described. It is probable, for example, that some of the extreme variations in nucleotide ratios reported for control liver PNA's are, in fact, reflections of varying levels and durations of stress to which such animals have been exposed prior to sacrifice, whether in slaughterhouse or laboratory.

Campbell and co-workers [15] have recently studied the effects of pregnancy, castration, and various gonadal substances on nucleic acid metabolism. They observed that in nonpregnant rats adrenalectomy, ovariectomy, or the injection of estradiol was accompanied by an increase in both the PNA and DNA of liver. Hypophysectomy reduced the PNA/DNA ratio, as previously observed by Geschwind, Li, and Evans [59], and prevented the effect of estradiol on such operated animals. In conformity with their original observation on the appearance of excess PNA in the livers of pregnant rats, they found that the injection of an alkaline placental extract produced an increase in the PNA of liver. This increase was much smaller, however, than that reported by them in pregnancy livers. These experiments indicate that such hormonal factors exert some direct and significant effect on either the rate of accumulation or synthesis of PNA. No clear inferences about the metabolic activities or potentialities of such a complex material as cellular PNA can be drawn from the bare record of such shifts in organ content or concentration, whether expressed in terms of presumed cell numbers on the basis of DNA-P estimates, or as total organ content.

Of the many accessory dietary factors, only vitamin B_{12} and folic acid have been directly implicated in the metabolism of the nucleic acids. In 1952 Lowe and his associates [90] reported that the rate of incorporation of ^{32}P into the PNA of liver is profoundly decreased in experimentally induced megaloblastosis of monkeys. The incorporation rate is greatly augmented by treatment with folic acid. Brown and Roll have recently summarized these and pertinent microbiologic studies [13] and have assigned to folic acid a relatively unique role as physiologic modulator of nucleic acid synthesis. They indicate that the inhibition of such synthesis by aminopterin, a folic acid antagonist, has been shown to result not from a specific interference with polynucleotide synthesis per se but rather from an interference with the incorporation of such active precursors as formate into the purines and into thymine. It is not surprising, therefore, that in vitamin B_{12} deficiency, which is closely related to folic acid deficiency, there should be alterations in the liver content of DNA and PNA reflecting an over-all reduction in the rate of cell division rather than any other specific effect [117, 120].

NEOPLASIA AND PNA METABOLISM. Early cytochemical studies of Caspersson and Santesson [19] drew attention to the possibility that morphological and chemical aberrations of the PNA-rich heterochromatic areas of the nucleus might be related not only to the intrinsic metabolism of malignant cells but also to chemical changes accompanying the transit from normal, compensatory, and functional growth processes to malignant transformation. More recently Moberger [102], using both ultraviolet and soft X-ray microabsorption techniques, found increased concentrations of cytoplasmic PNA in squamous cell carcinomas of vaginal mucosa in the mouse and also in the peripheral cell layers of infiltrating epidermoid carcinoma cords. He, however, cautioned that it would be impossible to infer from his results that the different quantitative distributions of PNA in infiltrating cancer cells as compared with non-neoplastic proliferating epithelial cells is a special characteristic either of the malignant process itself or of a transformation from non-neoplastic proliferation to malignant growth. Klein [83] has published a combined cytochemical and biochemical analysis of nucleic acid relationships in the free cells of ascitic fluid from a variety of induced peritoneal and ascites tumors. This type of tumor provides an ideal means for carrying out such studies. His analyses of the nucleic acid content of cells from a variety of tumors are reproduced in Table 7. The PNA to DNA ratio for most of his tumor cell representatives is substantially increased over that in nontumorous exudate cells, even where the DNA content per tumor cell is not significantly elevated. These studies on neoplasia suggest a distinct if undetermined metabolic role for PNA. The sober appraisal of Potter and Siekevitz [111] should be added, in conclusion, from their survey of recent chemical studies of neoplastic formation. They indicate that available data strongly point to the fact that neoplastic growths differ among themselves in metabolic patterns which result in nucleic acid synthesis, just as they believe that there may also be individual differences in metabolic patterns which control or fail to control growth generally. It is not yet entirely clear whether the most revealing insights of the future will come from a study of such individual differences or from an analysis of fundamental biological similarities. The broad trend of interrelated observations summarized in this report appear to favor the latter alternative.

5. *Summary and Formulations*

The clarification of the precise metabolic activities of PNA remains a major unresolved problem in cellular function. Two outstanding limitations on experimentation have hampered definitive research: (1) the inability until very recently to devise an *in vitro* system capable of reacting to PNA's in a specific way; this had made impossible a biochemical investigation of nucleic acids by those methods which have proved invaluable in enzyme chemistry; (2) the impossibility of demonstrating by feeding, injection, or deprivation techniques, any dramatic metabolic effects such as have provided the indicators for investigations of the metabolic roles of the hormones, vitamins, essential amino acids, and other factors. In this area research has, of necessity, been directed toward

TABLE 7. Nucleic Acid Content of Ascites Cells from Different Tumors[3]

Tumor	Number of Samples	Average Number of Cells × 10^6 per ml	Average Percentage T Cells	Of 100 T Cells		PNA/Total Cell Number 10^{-6} $\mu g\ P$[1]	(PNA/T Cell)[4]	DNA/Total Cell Number 10^{-6} $\mu g\ P$[2]	(DNA/T Cell)[4]	Ratio $\frac{PNA}{DNA}$
				Multinucleated	Mitosis					
15091a ca.	7	49.9	62.0	4.5	1.13	2.98	(5.02)	1.69	(2.63)	1.94
6C3HED lymphoma	7	148.5	95.5	1.4	0.8	0.82		0.61		1.30
dba lymphoma	9	174.6	94.3	0.2	0.4	0.73		0.74		0.97
Sarcoma 37	7	49.5	53.2	0.2	1.1	2.19	(4.77)	1.23	(1.88)	2.06
MC1M sarcoma	14	25.3	49.2	4.4	1.9	3.17	(7.18)	1.40	(2.71)	2.41
Yoshida sarcoma	7	80.8	82.6	0.2	0.85	1.25	(1.51)	0.88	(1.06)	1.45
Krebs 2 ca. (ref. 24)	14	142.0	83.0	0.7	2.5	2.16	(2.60)	1.14	(1.37)	1.81
Krebs lymphoma (ref. 20)	15	246.1	92.0			0.58		0.70		0.83
Ehrlich ca.	13	135.0	81.8	0.9	2.8	2.37	(2.92)	1.31	(1.64)	1.97
Non tumorous exudate cells (ref. 12)	15					0.44		0.77		0.59

[1] Corresponds to about 10.6 times more PNA.

[2] Corresponds to about 10.1 times more DNA.

[3] For errors of the method see reference below.

[4] Not calculated in cases of T (Tumor) cell frequencies higher than 90 per cent. The average PNA and DNA content of the single T cell lies between the value calculated per total cell number and that calculated per T cell. From Klein [83], Table II.

discovery of correlations of PNA activity with alterations in its type, composition, and cellular distribution, and with other recognized properties and activities of cells.

Chemical and physical studies reviewed in the first sections furnish solid answers to questions about the constituents of PNA's, their precise configuration and manner of interlinkage, but less clear-cut information about the probable sequence of mononucleotides in any given complex polynucleotide. They do not provide urgently needed evidence concerning the existence or structure of native PNA's or PNA-proteins within most living systems. Chemical and cytochemical investigations establish the widespread distribution and exact cellular localization of PNA's and bring into clear focus their remarkable lability in living, functioning organisms. Certain qualitative differences in the activities of PNA from nuclei or their microbiological analogues and cytoplasmic PNA's are recognized. These contributions, however, sketch in only the bare outlines of the picture.

Recent studies support the suggestion made many years ago by Spiegelman and Kamen [132] that PNA may channel energy into protein synthesizing reactions. Experiments on both microbiological and mammalian systems now establish the fact that, as Gale has put it [57], the pathway from amino acids to proteins, whether by exchange or synthesis, involves the confluence of those amino acids and proteins and the nucleic acids of the cell [61]. There is also good evidence that it is not so much preformed PNA's but rather those newly formed from available precursors which serve in some intimate if not essential capacity for supporting protein synthesis. A template mechanism of protein synthesis, dependent on stereo- and other specificities of cellular PNA's, is implied in Caspersson's hypotheses [18] and is more directly spelled out by Dounce [42], Bonner [6], and Gale [57]. Activity of a template must be recognized as the logical construct which it is, rather than as an established mechanism of protein formation. It is no more secure than the id, ego, and superego of the psychiatrist, though its proponents may be even more dedicated. Experimental systems for demonstrating, and even for manipulating [118], the operation of such supposed templates are now available in, for example, the resolved microbiological preparations of Gale and Folkes, the supplemented mammalian microsomal preparations of Siekevitz, and certain virus preparations. Past speculations have served their fruitful ends. The time is now ripe for experimental demonstration.

The complex interrelationships between the macromolecular DNA's of chromosomes, the unusually active PNA of nuclear material, and the physically and chemically labile PNA's of cellular cytoplasm have just recently come under investigation with techniques selective enough to permit accurate localization and yet sufficiently analytic to permit ultimate characterization of the materials studied.

Brachet recently emphasized [9] that "what is required now is the development of new methods for the isolation of native PNA's and decisive experiments for testing the biochemical and biological properties of these substances. Such experiments might well show that PNA, like DNA in the phage and transforming principle systems, plays an important genetic role and that it acts as a catalyst in protein synthesis." Data summarized in this review support this prediction and also indicate that the biological role of PNA may not, in fact, be restricted to a catalytic relationship to protein synthesis but may be that of a labile metabolite of fundamental importance for all metabolic processes involving phosphorylative mechanisms. Evidence for a possibly similar role for DNA [119] is still meager. Data are even now emerging which are consistent with the hypothesis that high-energy phosphate compounds or their monophosphate precursors may exist in the cell in unstable equilibrium with the pentose polynucleotides of organized and structured cell components.

With problems of such far-reaching significance so near apparent solution, this is a particularly unfavorable time to survey the metabolic activities of PNA. This is presented, nonetheless, with the underlying conviction that some of these problems will prove to be very nearly as complex as life itself.

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8. *Intracellular Lipides: Their Detection and Significance*

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THE PURPOSE of this chapter is to discuss some of the problems in the microscopic localization and characterization of lipides in animal tissues. The histochemistry of lipides is intrinsically more difficult and hence more primitive than the histochemistry of proteins and carbohydrates, simply because lipides as a class fail to exhibit sharply defined chemical properties and end-group reactions. Chemically the primary methods for distinguishing the various categories of lipides depend to a great extent on their differential solubilities in various organic solvents, procedures that are histochemically of negative rather than positive value.

The present review is divided into three sections: a brief consideration of the state of lipides in biological structures, discussion of some of the techniques currently available for identifying and characterizing lipides in histological preparations, and a final section on the significance of free lipide droplets in the cells of a few specific tissues. The section on methods is admittedly selective, since several extensive and competent evaluations have appeared within the past few years [8, 34, 88, 118, 121, 135].

1. State of Lipides in Biological Structures

As is widely recognized the histochemistry of lipides is generally restricted to the study of aggregates. Such aggregates, composed either entirely of lipides or of lipide-protein complexes that resemble lipides closely in their solubility properties (proteolipides [82]), are readily identified by their capacity to take up oil-soluble stains. Lipoproteins, in which the characteristics of the protein rather than the lipide dominate [82], present much more difficulty.

For the histologist the normal lipide-containing structures may be divided into three major classes [42]: (a) reserve lipides (for example, the droplets in the adipose-tissue cells), (b) functional lipides (for example, the droplets in adrenal cortical cells), and (c) structural lipides (for example, those conjugated with proteins, as in mitochondria). Ciaccio [42] finds it convenient to group the first two types as "metabolic" lipides and terms the last type "histolipides." Biochemists make a related distinction when they differentiate between "variable" and "constant" lipides [66, 114, 156]. For the biochemist the "constant" lipides are those that remain in the carcass of an animal that has died after prolonged inanition; in the mouse they constitute 2 per cent of the wet weight of the animal [103, 156].

To repeat, it is largely the "metabolic" or "variable" lipides that have received the attention of histochemists since it is these lipides that are aggregated and display the solubility and staining properties of lipides. Two categories of "constant" lipides also fall into this readily stainable class. One category consists of certain lipide droplets and proteolipides that are jealously retained by the organism even when it is dying of starvation. Examples for mammals are: (a) the myelin sheaths of axons and (b) the fat droplets in certain structural fat pads, such as those in the fasciae of the palms and soles, and of the cheeks of suckling infants.

A second category, pertinent to pathological conditions, includes lipides that are present normally in lipoprotein complexes but that have been released to coalesce and thus acquire lipide-staining characteristics [101, 124]. This process occurs during necrosis and autolysis and also in living cells ("fatty degeneration"). Chemically a tissue undergoing such fatty degeneration can be shown to contain no higher proportion of lipide than it did when healthy [158]. Histologically, however, the lipide is now readily detected. In actuality most instances of fatty change in cells fall into another category, namely, "fatty infiltration," in which lipide is derived from outside the cell rather than from the breakdown of its own cytoplasmic constituents. In the latter situation the percentage of lipide increases considerably above normal [67, 158]. Frequently "fatty infiltration" of normally nonfatty cells bespeaks an enzymatic disturbance [123].

The unmasking of previously invisible lipide is also termed "lipophanerosis." Over the years Ciaccio has been the major investigator of artificial lipophanerosis for demonstrating histo- or masked lipides. In one of these techniques, for example, mild protein hydrolysis with phenol or with pepsin followed by trypsin was used to reveal the lipides usually masked by protein in the Golgi apparatus [45, 155]. Heat has also been used for dissociating lipoproteins [15, 83]. In certain instances simply allowing unfixed tissue to autolyze will result in lipophanerosis. Alcohol, which disrupts phosphatide-protein complexes [39], has a similar effect [46]. The evident difficulty with such techniques lies in the probability that the lipides will form droplets or myelin figures and migrate from their original site [121].

By means of cell fractionation or even more indirect techniques chemical evidence has been adduced for considerable concentrations of lipides conjugated with protein in such structures as plasma membranes [52, 129], nuclei [166], mitochondria [154], and the Golgi apparatus [14, 146]. It remains probable that both the nature and the concentration of lipides in these structures vary with the cell type. The proportion of lipide in the plasma membranes of cells lying either in a tightly packed epithelium or in a viscous ground substance might be expected to differ from that in the membranes of free-living cells, such as erythrocytes or the eggs of marine echinoderms, which have been studied most extensively [52]. Furthermore, the empirical modifications of technique required for demonstrating the mitochondria and Golgi apparatus in different tissues, by methods that probably depend on the lipide component, suggest that the proportion of lipide varies in these organelles also.

It should be added that lipides in tissues, whether they are metabolic or structural, probably exist as mixtures—not only mixtures of different triglycerides (varying as to the length and unsaturation of their fatty acid moieties), but mixtures of different classes of lipides (triglycerides, phosphatides, steroids, glycolipides, sulfolipides). In many instances this point has not been established beyond doubt, since current extraction techniques rarely involve the initial separation of single objects, i.e. droplets or mitochondria or cell membranes. However, as noted above, a few such studies have been carried out (see also 106). Generally lipide analyses have been applied to extracts of whole tissue, such as the white matter of brain [81] or whole adrenal glands [105]. Nevertheless histochemical studies tend to confirm the conclusion that lipide-rich structures often contain a mixture of lipides rather than a single class (see p. 251, below). This mixing of lipides poses a considerable problem for the histochemist, because it may affect the sensitivity of certain tests (p. 242, below) or mask certain characteristics of individual classes, notably differential extractability, since lipides exhibit mutual solubility effects (p. 240, below).

2. *Methods for Demonstrating Lipides Histochemically*

With these considerations in mind let us turn to some of the methods now available for demonstrating lipides *in situ.* In this paper I shall attempt to employ the terminology currently used by lipide chemists [171]. Thus the word "lipide" ("lipid") refers to all classes of organic compounds that are relatively insoluble in aqueous media but generally soluble in organic and relatively nonpolar media such as alcohols, acetone, benzene, chloroform, ethers, and pyridine. The term is synonymous with lipoid as used by Cain [34]. The only subclasses considered here in any detail are the triglycerides, the phosphatides (phospholipides, lipines), the steroids, and the lipide-containing pigments. Less well-established methods exist for free fatty acids [118], glycolipides or cerebrosides [148], sulfolipides [170], and vitamin A and other carotenoids (lipochromes) [121].

Lipide aggregates or structures containing large concentrations of lipide can frequently be detected in the living or unfixed cell because of their distinctive refractivity. Such detection has been improved with the advent of the phase-contrast microscope [51]. Frequently, however, lipide-containing structures cannot be identified with certainty in fresh tissue because they are minute, because the optical properties or their surroundings are similar, or because the presence of protein masks the refractivity of the included lipide. Hence localization cannot always be made in fresh tissue of a lipide detected during subsequent manipulation [34].

The characterization of lipides, at least in the initial stages of an investigation, should always be carried out on completely unfixed tissue, since, as will be shown below, fixation affects several of their properties. Free cells can be utilized in smears; thin structures such as mesentery can be mounted directly on slides; cells in some other tissues may be examined in teased preparations. Most tissues, however, must be sectioned on the freezing microtome. To obtain even moderately good sections of unfixed tissue, the knife as well as the block of tissue should be chilled, as in the Adamstone and Taylor technique [1, 30, 165], or by installing the whole microtome in a Linderstrøm-Lang cryostat [49].

With such unfixed preparations particular care must of course be taken to avoid alterations due to atmospheric oxygen or intracellular enzymes.

EFFECTS OF FIXATION. Traditionally formalin (generally 10 per cent) is employed as the fixative when lipides are to be examined. Routinely the period of hardening is at least forty-eight hours, and it may be indefinite. The blocks are then sectioned on a freezing microtome, with or without the use of an embedding medium such as gelatin or gum arabic.

It has been insufficiently stressed that such fixation may alter lipides more or less radically (see, however, Halliday [95], Romeis [142], and Lison [121]). It seems to be generally assumed that exposure to formalin, which maintains cellular organization well [153], probably affects lipides but little. In routine histological or pathological investigation for the *presence* of lipide droplets, this assumption appears sufficiently valid. In histochemical studies on the *nature* of lipides, however, ample evidence has accumulated to disprove it.

TABLE 1. Effect of Fixation on the Mean Values for Total Lipide and Lipide Phosphorus in Adrenal Glands of Female Rats [105]

Adrenals (No.)	*Mean Gland wt., mg.*	*Total Lipide in Gland, %* *a*	*Phosphorus in Lipide, %* *b*	*Lipide Phosphorus in Gland, %* $a \times b/100$
Unfixed (50)	30.1	12.5	1.20	0.150
Fixed in neut. form. (50)	30.2	11.7 (−6%) *	0.98 (−18%)	0.115 (−23%)

* Figures in parentheses give per cent change from control values.

(1) One important consideration is that immersion of tissue in formalin allows the progressive destruction and removal of certain lipides, especially phosphatides. This fact has been noted by Halliday [95], Baker [9, 10], and Brante [25], among others. Table 1 presents some of our own data in support of this point [105]. In these experiments one adrenal from each of ten rats was put into a group that was extracted fresh, while the other was put into a group that was fixed for a week in 10 per cent neutral buffered formalin, washed overnight in running tap water, and then extracted. The adrenals (5 batches of each type) were extracted for total lipide by the improved chloroform-methanol

method of Folch *et al.* [81]. The table shows that in fixed glands the amount of total lipide extractable was insignificantly depressed in comparison to unfixed glands, but the amount of extractable lipide phosphorus was reduced nearly 25 per cent. (It is important to consider the possibility that formalin may render certain conjugated lipides difficult of extraction [10], although this effect has not been demonstrated specifically for the Folch method.)

Baker [9, 10], Cain [31], and others assert that the addition of calcium or cadmium ions to the formalin, a procedure long recommended by Ciaccio [42, 44], "restrains" the phosphatides from going into solution. Presumably the calcium forms salts with the phosphatides which are virtually insoluble in aqueous media. This assumption requires confirmation. For example Karnovsky and I found in a single comparison conducted in the same way as those cited above that immersion of adrenals in neutral calcium-formalin allowed nearly half as much loss of extractable lipide phosphorus as did immersion in neutral formalin lacking calcium.

Still more suggestive, though indirect, evidence that addition of calcium to the fixative fails to retain all phosphatides derives from the histological appearance of sections prepared according to Baker's method [10]. It is general experience that even small blocks of tissue show stainable phosphatides only near the surface; the interior of the block may contain little or no stainable material. For example, in the adrenal gland the mitochondria in only the glomerulosa and outer fasciculata are stained consistently; those in the inner fasciculata and medulla are generally unstained [34, 35].

Further evidence for the destruction of phosphatides in the presence of calcium has been adduced by Dr. D. W. Fawcett (1949, unpublished). Rats killed with illuminating gas were perfused briefly through the heart with (a) saline, (b) saline followed by calcium-formalin, and (c) saline followed by a calcium-formalin-potassium dichromate mixture. Small blocks from various organs were then excised, placed in calcium-formalin, and thereafter treated exactly according to Baker's method for demonstrating phosphatides [10]. Only with treatment c were stainable materials retained in normal-appearing structures throughout the blocks. In preparations from a and b, stainable materials were reduced in over-all amount and often coalesced (Figs. 62, 63). These results certainly suggest that initial exposure to calcium-formalin alone does not serve to "restrain" phosphatides, whereas the addition of dichromate to the initial fixative may be effective [43, 45]. As frequently stressed by the Baker school (see [34]), however, such treatment might render compounds other than phosphatides stainable, so that the specificity of this variant must be confirmed by model tests.

Particular emphasis has been laid on the destructive effects of formalin for plasmalogen. Such acetal phosphatides cannot be identified in tissues that have been fixed for more than a few hours [32, 33, 99, 132, 137]. The precise manner in which these or other phosphatides are destroyed has not been established.

(2) Another important consideration in the use of fixed tissue for the study of lipides resides in the demonstration that soaking the tissue in any aqueous medium permits the development of aldehydes through the autoxidation of the unsaturated fatty acid residues in the lipides [105]. It seems probable, furthermore, that careful study would show that free fatty acids and lipide peroxides develop at the same time. The normal occurrence of significant and detectable quantities of any of these products of rancidity in fresh tissue seems generally unlikely [126], although traces appear to be present in certain lipide pigments (p. 244, below).

In the last fifteen years interest in the carbonyls detectable in the lipide droplets of fixed tissues was stimulated by the conclusion, fostered principally in the Department of Anatomy at Harvard Medical School, that these carbonyls are ketonic and might represent unmasked ketosteroids when found in the droplets of steroid-producing cells [16, 61, 64, 65, 93]. Evidence soon accumulated, however, that the carbonyls are probably aldehydic rather than ketonic, since they stain with the Schiff reagent (a bisulfite reaction, essentially limited to aldehydes) as well as with hydrazines (a Schiff-base reaction characterizing both aldehydes and ketones) [2, 23, 32, 33, 47, 87–89, 120, 121, 132]. It was originally suggested [2, 87] that the aldehydes are derived from plasmalogen, but later work [32, 33, 89, 132] indicated that they are secondary, or artifactual products arising from the autoxidation of double bonds in the fatty acid moieties of the lipides. Thus droplet reactivity cannot be demonstrated in fresh unfixed tissue even after treatment with mercuric chloride or other agents that split the acetal linkage, but it develops after exposure to oxidizing agents or immersion in media that permit autoxidation [56]. Cain has coined the term "pseudoplasmal" for such artifactually produced lipide aldehydes. It should be mentioned that earlier histochemists, in the course of studying the distribution of true plasmalogen, had also postulated such autoxidative products as the cause of droplet reactivity [136, 137, 159, 161]. In the European literature the test for true plasmal (p. 241, below) is often termed the Feulgen-Voit reaction [80], whereas the procedure for revealing secondary or artifactual aldehydes is called the Feulgen-Verne reaction.

Karnovsky and I [105] have recently adduced what appears to be firm chemical evidence for this hypothesis that the lipide aldehydes are products of autoxidation of unsaturated fatty acids. We found that lipides extracted from fresh adrenals and from fixed adrenals (10 per cent neutral buffered formalin one week, overnight wash) differed significantly in three respects (Table 2). In the lipides from fixed adrenals, the iodine value and the titre of polyene fatty acids with two to five double bonds were significantly lower while the concentration of lipide aldehyde was doubled, in comparison with lipides from fresh adrenals. These chemical changes occurred concomitantly with the development of carbonyl reactivity in the droplets (Fig. 64). We then found that the addition to the fixative of a trace (0.1 per cent or less) of any one of the phenolic antioxidants used by the fat and oil chemists [104], such as pyrogallol, hydroquinone, or nordihydroguaiaretic acid, entirely prevented the development of carbonyl reactivity (Fig. 65). Such treatment likewise prevented the chemical changes described above (Table 2, line

TABLE 2. Mean Figures for the Iodine Value, the Titre of Polyene Fatty Acids, and the Amount of Aldehyde in the Lipides Extracted from 50 Unfixed Rat Adrenals, from 50 Adrenals Fixed for a Week in 10 Per Cent Neutral Buffered Formalin (NF), and from 30 Adrenals Fixed for a Week in 10 Per Cent Neutral Buffered Formalin Containing 0.1 Per Cent Hydroquinone (NF + HQ) [105]

Adrenals	*Iodine Value*	*Titre of Polyene Fatty Acids* *	*Aldehydic Lipide* ** *Per Cent*
Unfixed	116	205	1.9
Fixed, NF	90	165	3.8
	(−22%) ***	(−20%)	(+100%)
Fixed, NF + HQ	112	204	1.2
	(−3%)	(−0%)	(−35%)

* $E^{1\%}_{1\ \mathrm{cm.}}$ at 233 mμ after conjugation of the double bonds with alkali.

** Expressed as acetalphosphatide.

*** Figures in parentheses give per cent change from control values.

3). Perhaps the decline from normal in the amount of aldehydic lipide that we observed after fixation in formalin containing 0.1 per cent hydroquinone may reflect the destruction of true plasmalogen during fixation. Hence the amount of *new* aldehyde formed is probably higher than that indicated in line 2.

It was also found that histochemical reactivity to carbonyl reagents was depressed, although not entirely inhibited, if a trace of a chelating agent such as dithizone or 8-hydroxyquinoline was added to formalin. This finding suggests that metals are catalysts in the autoxidation of the lipides.

Histochemical preparations of such glands revealed that adrenals fixed in the presence of an antioxidant under no conditions developed hydrazide reactivity [6] when Schiff reactivity was suppressed. Hence the suggestion [56, 61] that detectable quantities of ketonic lipide (ketosteroid?) might be "unmasked" during fixation seems ruled out. Deane and Andrews [56] demonstrated that when fresh sections of ovary were treated with halogens, the droplets became reactive to hydrazide without becoming reactive to the Schiff reagent. We postulated that the carbonyls appearing in such preparations were unmasked ketones, detectable because the usual production of pseudoplasmal had been blocked. By means of test-tube experiments, however, Karnovsky [105] demonstrated that halogens are capable of oxidizing cholesterol to a ketonic form while at the same time converting any aldehydes to unreactive compounds (acids?). He thus showed that the ketones demonstrated by Deane and Andrews were probably also artifactual.

Lest it be thought that such changes in lipides during fixation are limited to formalin, it should be added that Gomori [89] and Karnovsky and Deane [105] demonstrated that any of a number of aqueous fixatives allow the development of droplet carbonyl reactivity (Figs. 66, 67). In some instances reactivity appeared even greater than when formalin served as the fixative, the latter being a mild reducing agent [56]. In all probability it is atmospheric oxygen rather than the fixative *per se* that permits autoxidation.

(3) Numerous additional artifacts have been reported to occur in lipide aggregates as a result of fixing the tissue. The tendency of some of the constituents, notably cholesterol esters (Fig. 71), to crystallize is well known [47, 73, 121, 172]. Dissociation of lipoprotein structures may also occur.

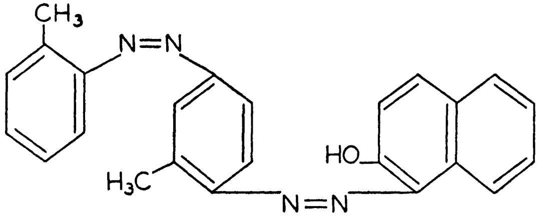
Another alteration is the occasional distortion of the shape of lipide droplets. Distortions are particularly demonstrable when osmium tetroxide is used as the fixative. The extreme variety in shape produced by such fixation has especially intrigued Dr. D. W. Fawcett, who kindly provided me with the accompanying electron micrographs (Figs. 68, 69). These two samples of frog tissue were fixed in Palade's [134] buffered osmium tetroxide medium and treated alike thereafter. Yet in the "cortical" interrenal cell, the droplets have remained nearly spherical, whereas in the liver cell they have assumed bizarre myelin forms. Intermediate shapes have been found in other tissues. It seems probable to us that this response in shape of the droplets reflects primarily the nature of the lipides, although the chemistry and viscosity of the surrounding protoplasm may play some role also.

SUDANOPHILIA. Just as solubility in organic media remains the fundamental method of the chemist for identifying lipides, so the uptake of a nonpolar stain is the fundamental method of the histochemist. The more familiar of these stains are sudan III (orange), sudan IV (orange-red), oil red O, and sudan black B. Of these four the first three are derivatives of beta-naphthol [118], and sudan black B is now thought to be a derivative of naphthyl diamine [119] (Text Fig. A). For the first class Michaelis [128] adduced considerable chemical evidence that internal bonding prevents any chemical reactivity and that their ability to color lipides depends on a favorable partition coefficient between the lipide and the solvent. It is generally asserted [88, 121], therefore, that the specificity of these stains for lipides is absolute. Several pitfalls appear to exist, however, for the histologist who assumes that he is thereby demonstrating all lipides, on the one hand, or only lipides, on the other.

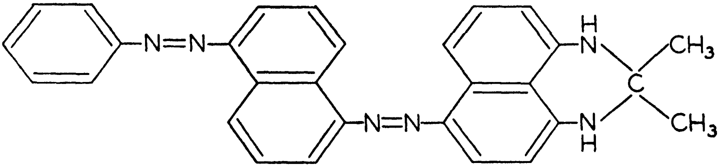
(1) The first problem is the danger that the solvent used for the stain may extract some of the tissue lipides. Because of the nonpolar nature of these stains organic solvents are generally required. Some investigators [90] have experimented with the use of suspensions of sudans in aqueous media, but few others have adopted such procedures because staining is slow and the sudan tends to precipitate on the section.

The traditional staining baths are saturated solutions of sudans in 70 per cent ethanol or in a 1:1 mixture of 70 per cent ethanol and acetone. By comparison of the lipide content of sections before and after staining [121, 142], or merely by inspection of the section during staining, it has been well established that some lipide droplets, particularly small ones, are removed during the staining period. In consequence a number of workers have experimented with solvents that dissolve sufficient amounts of stain but remove little lipide. I have no personal experience with the use of such carriers, but diacetin (glycerol diacetate) [113], ethylene or propylene glycol [41], triethylphosphate [88], as well as supersaturated solutions of 40 to 50 per cent ethanol [142] or isopropanol [116, 118] are all reported to yield superior results.

Sudan IV



Sudan black B



TEXT FIGURE A. The chemical structure of sudan IV [128] and of sudan black B [119]. Sudan IV, like most oil-soluble stains, possesses a free phenolic hydroxyl group. Sudan black B, like a few of these stains [118], possesses secondary amino groups.

(2) To determine whether the stained structure is indeed a lipide, controls are necessary. The usual control has been to test the stainability of a section previously immersed in a lipide solvent such as absolute ethanol, acetone, pyridine, or chloroform. In many instances, however, this control has been omitted, first, because of Michaelis' [128]

conclusion that the sudan stains are incapable of true dye-binding, and second, because some lipides are extractable only with great difficulty. The latter statement applies in particular to the lipide pigments (p. 243, below). In order to overcome the second difficulty Lillie [118, 119] has devised another type of control in which the stained section is exposed to fresh solvent, into which the stain should pass rapidly if staining is indeed physical rather than chemical. Lillie claims that if a sufficiently mild solvent (for example, a glycol) is used, the stain can be repeatedly removed from and restored to true lipide structures.

It was by employing the latter type of control that Lillie and Burtner [119] first drew attention to the anomalous behavior of some structures toward sudan stains. They studied in particular the granules of neutrophilic leucocytes, which take up sudan stains [100], but generally only when staining is prolonged and carried out above room temperature. Following staining, the granules decolored with the greatest difficulty. Lillie and Burtner found that they could not then be re-stained, as could myelin and other truly lipide structures. To test the possibility that the hydroxyl group on the red dyes or the secondary amino groups on sudan black B might be entering into chemical union, they tested such stains in which these groups had been acetylated or benzoylated. With these inert compounds true lipide structures stained readily, but the neutrophil granules could not be stained, even with prolonged exposure at high temperature. Lillie and Burtner concluded that the sudanophilia of the granules therefore depends not on physical dissolution of the stain, but on true dye-binding through the hydroxyl or secondary amino groups. In this particular example the stained structures appeared to be nonlipide.

Casselman [38] has confirmed the hypothesis that sudan black B can bind chemically to tissue structures. Since sudan black becomes bound to pure lecithin [148], however, the stainable substance may sometimes be lipide in nature, rather than nonlipide, as in the previous example.

(3) A final consideration under the topic of sudanophilia involves the physical state of the lipide being tested. Cain [34] emphasizes that oil-soluble stains are ineffective for revealing lipides in the solid state. It is of course demonstrable that crystallized lipides remain unstained (Fig. 70; see also Yoffey and Baxter [172], their Fig. 17). Nevertheless, it is common experience that certain presumably solid lipides, such as the lipide pigments (below, p. 243), are readily stainable (Fig. 74). Such pigments consist of oxidized and polymerized lipides and are

insoluble and apparently solid, yet they consume sudan stains, as do mitochondria and myelin after chromation [42, 69]. Perhaps the carrier for the stain acts somewhat as a paint softener to permit some penetration.

PHOSPHATIDES. Phosphatides consist of an alcohol, fatty acids or a fatty aldehyde, phosphoric acid, and a base. The principal examples are lecithin (phosphatidyl choline), the cephalins (phosphatidyl ethanolamine and phosphatidyl serine), inositol phosphatides, the sphingomyelins, and plasmalogen [171]. Phosphatides apparently occur principally in the structural or histolipides (i.e. mitochondria, cell membranes), in contrast to triglycerides, which occur most abundantly in free droplets. In general they contain more highly unsaturated fatty acids than do triglycerides [22]. The fatty aldehydes of plasmalogen, however, appear to be entirely saturated.

(1) The exact mechanism of the staining of phosphatides remains in question. Review of the methods available for demonstrating this class (excluding plasmalogen), however, strongly suggests that staining depends on the presence of the phosphate moiety. The established methods [10, 43, 45, 69], all derived from the classic technique of Smith and Dietrich, have the following features in common: the tissue is fixed in formalin or potassium dichromate, postchromated for about two days, overstained with hematoxylin (hematein), and then vigorously destained. In some methods [10], frozen sections are employed, in others [45], paraffin sections. Baker [10, 11] conducted model tests with presumably pure compounds and found that only highly acidic compounds like phosphatides, nucleic acids, and acid mucopolysaccharides were stained by his variant of the method. A control preparation extracted with hot pyridine before mordanting and staining distinguishes the lipides from nonlipides. (A nonchromated control may be added to indicate whether any staining in the pyridine-extracted control is due to metal-containing compounds, like hemoglobin, that are capable of forming hematoxylin lakes directly.)

The model tests performed by Baker [10, 11] and Casselman [37] have demonstrated satisfactorily that Baker's procedure, if carried out according to directions, is highly specific. Unless chromation is unduly prolonged so that the double bonds of unsaturated fatty acids bind some chromium, the staining appears to be limited to the phosphate moiety. Two lines of evidence indicate this. Ciaccio [43] demonstrated that the chromium-containing lipides isolated from material so prepared contained phosphorus and chromium in 1:1 ratio. Casselman [37] found that intense staining could be obtained with synthetic lecithins containing only *saturated* fatty acids. Thus Lison's [121] hypothesis that it is the highly unsaturated fatty acids characteristic of naturally occurring phosphatides that are responsible for binding chromium seems unlikely. Parenthetically, however, it seems probable to me that the still ill-defined class of sulfolipides might also react.

While the specificity of the Baker test for lipides containing a phosphate (or possibly sulfate) moiety appears established, the sensitivity of the method remains to be determined. I have considered above (p. 232) the likelihood that considerable phosphatide is destroyed during the period of fixation. Perhaps that possibility explains the following finding. Karnovsky *et al.* [106] observed that no structures whatever in the starfish gut stained by the Baker method, yet the lipide contained approximately as much phosphorus as the lipide extracted from the adrenal gland [105]. In the adrenal gland, however, the abundant mitochondria of the cortical cells stain intensely. The only peculiarity of the starfish lipide that was discovered was that phosphorus composed only 2.5 per cent of the isolated phosphatide rather than 4 per cent, as in lecithin.

The methods under consideration, of course, do not permit distinguishing between the several types of phosphatides. Removal of individual phosphatides by solvents before chromation does not seem feasible, since there are only slight differences in solubility among pure lecithin, cephalins, and sphingomyelins. Furthermore, when such compounds occur in mixtures, as they seem to do [81, 154], differential solubilities become modified. In addition, the insolubility in acetone of pure phosphatides is overcome whenever they occur mixed with triglycerides. The latter fact makes confirmation of the identification of phosphatides in droplets consisting largely of triglyceride a difficult matter. For example, results with the Baker method on the adrenal gland suggest that some of the cortical droplets contain phosphatides [105]. Yet because of mutual solubility effects when phosphatides occur with triglycerides, these putative phosphatides are removed if the tissue is extracted with acetone before chromation. It would seem important, therefore, to employ methods depending on other moieties of the phosphatide molecule to confirm such results. Landing *et al.* [112] have introduced a method utilizing the reaction between choline and phosphomolybdic acid for identifying lecithin and sphingomyelins, but as yet I have seen no reports of comparison of this technique with the Baker method.

(2) A consideration of phosphatides would be incomplete without brief mention of the plasmalogens. These phosphatides are thought to contain a fatty aldehyde in acetal linkage with glycerophosphorylethanolamine [79, 157]. As noted above (p. 233), the principal interest that these compounds have held for histochemists resides in the frequency of false-positive results. This difficulty arises from the fact that an aldehyde reaction is employed for their identification, after splitting the acetal linkage by brief exposure to mercuric chloride or dilute mineral acid. Unless completely fresh tissue is used, unless exposure to both the hydrolyzing agent and the Schiff reagent is kept brief, and unless an untreated control section is employed, aldehydes other than those derived from plasmalogen may be responsible for staining. A control section first extracted with an appropriate lipide solvent is necessary to establish the lipide nature of the stained substance. When carefully demonstrated, "true" plasmal is limited to the homogeneous, background cytoplasm and does not occur in droplets, as does pseudoplasmal [56, 94, 132, 137].

True plasmalogen appears to be generally, though not invariably, more abundant in nonfatty tissues than in fatty ones [94, 132, 137]. Thus in the adrenal gland it is more abundant in the medulla than in the cortex. It is reported to be rich in the cytoplasm of brown-adipose cells, however [76].

STEROIDS. The only steroids apparently present in detectable quantities in animal tissues are cholesterol and its esters. These are abundant in the glands that produce steroid hormones, in sebum, in nephrotic kidneys, in gall stones, and in atheromatous plaques of blood vessels. Traces occur in the structural lipides of all tissues. The presence of esterified cholesterol may be surmised from the tendency for crystals to form during fixation [47]; these are readily detected with the polarizing microscope (Fig. 71). But lipides other than cholesterol esters are also capable of crystallizing during fixation [121] (see Fig. 70). It is possible, furthermore, to identify beta-hydroxysterols, of which cholesterol is the most abundant representative, by precipitation with digitonin [16, 121].

The only relatively specific techniques for demonstrating cholesterol together with its esters are the Schultz methods [147], which depend on the ability of delta-5-polycyclic compounds to oxidize to 7-oxy compounds under the influence of atmospheric oxygen, ferric ammonium sulfate (iron alum), or acetic anhydride. The oxidized compounds give a blue-green color when treated with concentrated sulfuric acid. Hershberger [118] has found that hydrogen peroxide or sodium iodate are more rapid oxidizers, and Dr. M. H. Burgos (1953, personal communication) has shown that the periodic acid has the same property. Although some other unsaturated, polycyclic compounds may, under these conditions, give brown or purple pigments, the only substances that have been found to give conflicting blue-green colors by *in vitro* tests are certain carotenoids and vitamin A [34, 108, 118]. Presumably the presence of these could be determined by their characteristic fluorescence or by specific color reactions. It is noteworthy, however, that carotenoids are sometimes abundant in the lipides of steroid-producing cells (for example, lutein cells).

The limitation of the Schultz test lies, however, not in its specificity, which is high, but in its sensitivity, which is low. Both Everett [74] and Reiner [139], by testing *in vitro* the influence of diluting cholesterol with other lipides, have shown that unless the steroid constitutes approximately 10 per cent of the mixture, no color is detectable. Furthermore, in animal tissues, seemingly minor changes in the percentage of steroid cause striking differences in histochemical reactivity. Table 3 shows the results of an unpublished experiment by Karnovsky and me, in which the cholesterol content of adrenal lipide was determined chemically by the method of Zlatkis, Zak, and Boyle [110, 174] and histochemically by the Schultz method, with iron alum as the oxidizing agent. One group of rats served as controls; the other group was exposed to cold (3°C) for eight days. As can be seen, a drop in the percentage of cholesterol in the lipide from about 28 in the "normal" adrenals to about 24 in the "stressed" adrenals resulted in virtual loss of Schultz reactivity (Figs. 72, 73). Unless it be argued that most of the residual steroid in the

TABLE 3. Cholesterol Content [174] of the Adrenals from Control Female Rats and from 10 Rats Exposed to Cold for 8 Days, as Compared with the Schultz Reactivity of the Lipide Droplets (Figs. 72, 73)

Rats	*Mean Gland wt., mg.*	*Lipide in Gland, %*	*Cholesterol in Lipide, %*
Normal	26.2	12.5	27.8
Stressed	38.8	9.7	23.5
	(+57%) *	(−22%)	(−15%)

* Figures in parentheses give per cent change from control values.

adrenals of the "stressed" rats occurred in structural lipides rather than in the cortical droplets, this result only serves to underline the lack of sensitivity of the Schultz method. For the histophysiologist, of course, lack of sensitivity might be advantageous in certain situations, since differences between groups are thereby heightened.

LIPIDE PIGMENTS. In certain types of mammalian cells, particularly cells of aged animals, one may find detectable quantities of insoluble, lipide-containing, yellow-to-brown pigments. The names that have been assigned to them are many—*Abnutzungspigmente,* wear-and-tear pigments, chromolipoids, lipofuscins, brown degeneration, luteolipin, ceroid, cytolipochrome, etc.—and their characteristics vary not only with the tissue but with the species. Although it may be important for the pathologist to emphasize their distinctive properties, certain generalizations about the group appear valid.

The lipide content of such pigments is attested by their sudanophilia, even with the inert dyes prepared by Lillie and Burtner [119]; yet they are resistant to extraction by lipide solvents and to paraffin embedding. Their insolubility apparently results from prolonged autoxidation of unsaturated lipides, causing peroxidation of double bonds and the condensation of these oxidized forms into viscous or solid polymers [98, 107, 138]. It is such characteristics that make polyunsaturated lipides useful for making linoleum and as paint-drying oils. It seems probable that the pigmentation depends on the emergence of conjugated double bonds [104, 107], a property also suggested by the fluorescence of the granules in the ultraviolet.

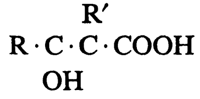
Normally, the occurrence of natural antioxidants such as carotenoids [160] or alpha-tocopherol [104, 107] probably inhibits such oxidative processes. In certain pathological states, however, these substances may be absent or their effectiveness overcome, so that autoxidation of intracellular lipides occurs. Choline deficiency was early recognized to cause the deposition of ceroid in the hepatic and Kupffer cells [71]; a related lipofuscin develops in muscle fibers and adipose-tissue cells in vitamin E deficiency [70, 91, 126a]. The aging of steroid-producing cells such as lutein cells or cells lying in the reticularis of the adrenal cortex may induce the appearance of "brown degeneration" [40, 58]. The presence of pro-oxidants like hemoglobin may also stimulate such changes [104], and in some circumstances iron is detectable among the pigment granules [85, 115]. Both Hartroft [97] and Casselman [36] have demonstrated *in vitro* that an insoluble fatty pigment develops when unsaturated lipides are incubated with erythrocytes or hemoglobin. In such models, alpha-tocopherol or hydroquinone were effective in inhibiting the process.

As a general rule, the reactions given in common by such lipide pigments wherever they occur appear to be the following: (a) *sudanophilia,* even following prolonged extraction of the tissue with fat solvents (Fig. 74) (some pigments that have existed for long periods of time lose their sudanophilia [135], possibly because they have become too solidified—see above, p. 238); (b) a *peroxide reaction* [86] when the pigment is of relatively recent origin; (c) *basophilia* and (d) *acid-fastness,* suggesting the presence of free fatty acids; and a number of properties all indicating the presence of double bonds, some conjugated: (e) *pigmentation,* (f) *fluorescence* (Fig. 75), and (g) an *aldehyde* (Schiff) *reaction* following oxidation with chromic acid (Fig. 76) or performic acid [117]. It may be concluded that such pigments contain a complex mixture of lipides in various stages of autoxidation [175]. Proteins also may be included within the pigment mass.

Whenever such pigments occur in the cells of steroid-producing organs, they also contain detectable quantities of phosphatides and cholesterol (Fig. 77) [40, 58]. In all probability this admixture reflects the normal presence of these particular lipides in the droplets of such cells (below, p. 251), so that they are trapped when oxidation occurs. Wherever they are found, in steroid-producing or other types of cells, the masses of lipide pigment appear highly heterogeneous when examined with the electron microscope (Figs. 78, 79).

Many of the above characteristics have likewise been demonstrated for histolipides in tissues that have been subjected to oxidation by dichromate, and possibly all of them could be detected. For example, after chromation the sudanophilia of mitochondria and of myelin persists even when the tissue has been embedded in paraffin [42, 69]. Furthermore, under such circumstances, mitochondria, at least, appear also to be acid-fast, if the Benda method [50] can be so interpreted.

Further consideration of acid-fastness is in order, since this property, too, has been the subject of considerable research in recent years. It depends on the ability of the substance to withstand destaining by acid alcohol following staining by a basic dye (for example, the Ziehl-Neelsen carbol-fuchsin technique [50]). The acid treatment completely destains most basophilic objects such as nuclei and mucins. Recently, Berg [19] has demonstrated that there are degrees of acid-fastness, since the capsules of tubercle and lepra bacilli and the neck pieces of spermatozoa are acid-fast after staining with a number of basic dyes including crystal violet, whereas ceroid and some other substances that are acid-fast by the Ziehl-Neelsen test fail to retain crystal violet. Berg [20] then adduced evidence that the marked acid-fastness characterizing the first group depends on the presence of mycolic acid or a close relative.

Chemical evidence indicates that mycolic acid ($C_{88}H_{176}O_4$) is a large, branched hydroxy fatty acid with the structure  $R\cdot\underset{OH}{C}\cdot\overset{R'}{C}\cdot COOH$ [7, 68]. Asselineau and Lederer [7] postulate that it is the presence of a hydroxyl group on the carbon beta to the carboxyl group that endows the free acid with its tenacious affinity for basic dyes. They have not been able to demonstrate to their own satisfaction, however, that sufficient acid occurs in unesterified form to account for the acid-fast reaction of intact tubercle bacilli.

3. Biological Significance of Intracellular Lipide Droplets

Free lipide droplets may be present in many different cells and tissues of the mammalian body. In general, droplets are restricted to the cytoplasm, though they occasionally occur in the nucleus [27]. In the present paper I have chosen to limit the discussion of the biological significance of cellular lipide droplets to the three tissues in which they have been studied most extensively, namely the adipose tissues, the hepatic parenchyma, and the steroid hormone-producing tissues.[1]

ADIPOSE TISSUES. The adipose tissues are concerned principally with the storage of lipides as fuel reserves for the whole organism [66, 163] (see, however, above, p. 228). While there appears to be continual chemical "turnover" of lipides in the depots, gross mobilization (withdrawal) occurs only in conditions that involve conspicuous catabolism of lipides.

In most mammalian species two distinct types of adipose tissue exist

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[1] Aside from these, other tissues that may normally display free lipide droplets include the epithelial cells of the sebaceous glands [131], mammary glands [62], apocrine sweat glands [29], intestine [12], renal tubules [141, 151], oviduct [55], uterus and cervix [169], and male reproductive tract [168], plus striated muscle fibers [28], chondrocytes [130], and macrophages [141]. Pathologically, cells in other tissues may also undergo fatty infiltration.

—unilocular (white) fat and multilocular (brown) fat. In adult human beings in good nutrition, all fat cells appear to be of the first sort, but in infants or in adults suffering from emaciation, some lobules of brown fat also occur. Brown fat is by no means limited to the hibernating mammals, as suggested by its misnomer, the "hibernating gland." The histochemical and physiological differences between these two classes of adipose tissue have received considerable attention since the Wertheimer and Shapiro review [163] and will be the subject of the present discussion.

White-fat cells are normally extremely large (about 100 μ in diameter) and are spherical if isolated but compressed into polygonal shapes if crowded together (Fig. 70). They contain a single droplet of lipide (hence unilocular), and the cytoplasm is restricted to a thin, submembranous rim (Fig. 80). Brown-fat cells are smaller (about 30 μ in diameter), polygonal in shape, and epithelioid in arrangement. Each cell contains numerous small lipide droplets (multilocular), and cytoplasmic strands extend between the droplets (Fig. 80).

Histochemically, the lipide droplets in both types of adipose tissue appear to consist simply of triglycerides, since phosphatides (Fig. 81), cholesterol, and free fatty acids are not demonstrable [76, 127]. In all likelihood, the phosphatides and cholesterol found in extracts from these tissues are derived from the structural lipides, and it might be expected that they would be more abundant in extracts of brown fat because of the abundance of mitochondria therein (Fig. 81).

The nature and location of the pigment in brown-fat cells that produces the distinctive coloration of the gross tissue remain undetermined. Fawcett's [76] suggestion that it might be attributable to a mitochondrial pigment such as reported by Bensley [17] for isolated liver mitochondria now seems less likely in view of Bensley's subsequent finding [18] that the pigment was hemoglobin adsorbed by the mitochondria during homogenization of the unwashed tissue. To date, no biochemist appears to have separated the lipide and other fractions of brown fat in order to locate and characterize the pigment.

Present evidence suggests that the most conspicuous chemical distinction between these two classes of adipose tissue is that the triglycerides of brown fat contain more highly saturated fatty acids than do those of white fat. This conclusion is based on Fawcett's [76] observation that (a) primary osmication of the droplets fails to occur in brown fat cells whereas it occurs readily in white fat cells, and (b) lipides expressed from brown-fat cells crystallize much more readily at room temperature than do those from white-fat cells. Very little direct chemical evidence for this hypothesis is available. However, Coninx-Girardet [48] reported that for a single marmot the iodine values of the lipides from brown and white fat were, respectively, 71 and 90. Dr. C. P. Lyman kindly determined the values for these two types of fat in the Swiss mouse and obtained average values of 74 and 80 (three animals). Indirect confirmatory evidence is provided by the responses of the two types of lipide to the pseudoplasmal test. I compared mouse brown- and white-adipose tissue fixed in formalin, washed overnight, and then immersed in Schiff reagent for thirty minutes. The lipide droplets of white fat fixed for four days developed a faint pseudoplasmal reaction and a more distinct, although still moderate reaction when fixed for 8 days. Not even with this longer period of fixation did the droplets of brown fat show any reaction whatsoever. Parenthetically, this difference in the degree of unsaturation of the lipides in white and brown fat probably accounts for the fact that only white-adipose tissue deposits lipofuscin [126a], since such pigments arise from the autoxidation of unsaturated lipides.

Even white-fat lipides generally fail to develop a conspicuous pseudoplasmal reaction when fixation has been relatively short, and they thus contrast sharply with adrenal cortical cells (Figs. 83, 84). In rats fed laboratory rations, the iodine value of white-fat lipide is 89 [77], whereas that of adrenal lipide is 116 (Table 2). Prolonged fixation, of course, produces an intense aldehyde reaction of the lipide droplets of white-adipose tissue [132], although both Dempsey *et al.* [62] and Fawcett [76] have noted that individual fat cells vary greatly in this respect. It might be valuable to explore the usefulness of the pseudoplasmal reaction as an index of lipide unsaturation by comparing the adipose-tissue cells in normal animals with those in animals in which unsaturation of the lipides has increased. Desaturation of the depot lipides has been reported for the hamster when exposed to cold [77] and for several other species with administration of thyroid hormone [22].

The biochemical distinction between these two classes of adipose tissue resides in the higher metabolic activity of brown fat [26]. Histochemically, the cytoplasm of brown-fat cells displays higher concentrations of both oxidative and esterolytic enzymes than does that of white-fat cells [76, 127]. These concentrations suggest that brown fat possesses a greater capacity to metabolize lipides or precursor substances. A variety of conditions that favor lipogenesis, such as the administration of insulin or refeeding a fasted animal, cause the deposition of glycogen in both types of cells (Fig. 82). Glycogen becomes much more abundant, however, both chemically and histochemically, in brown-adipose than in white-adipose tissue [72, 75, 76, 140]. Apparently glycogen is stored in the cytoplasm of fat cells prior to the local synthesis of lipides from carbohydrates [21, 163].

Dr. Fawcett has suggested to me that the smaller droplet size in brown-fat cells may be a direct reflection of the fact that there is greater turnover of lipide in this type of adipose tissue. This hypothesis is given weight by the observation that brown-fat cells become unilocular and nearly indistinguishable from white-fat cells after denervation [150], which appears to depress the mobilization of their lipides. (For further comments on the significance of droplet size, see below, pp. 250, 252.)

Additional distinctions between the two classes of adipose tissue have been observed with respect to their responses to fasting, hormonal imbalance, vitamin deficiencies and other disease states [3, 26, 76]. Neither the differences in metabolic activity between the two types nor the distinctions in response to over-all bodily state, however, permit us at this time to draw any conclusion as to the particular function of brown fat.

HEPATIC PARENCHYMA. In its role as the controlling metabolic organ of the body, the liver plays a predominant part in the utilization and also the synthesis of lipides. This is the principal site of hydrolysis and oxidation of lipides to 2, 3, and 4 carbon units. In addition, even more than adipose tissue, the liver converts dietary carbohydrate to lipides. Whenever there is an especial call for the use of the depot lipides as fuel, or whenever the hepatic-cell enzymes are incapable of disposing of lipides so rapidly as they are brought to the liver or synthesized there, a "fatty liver" ensues [152]. The conditions producing this state are legion [66, 102, 164], but much remains to be learned of the basic factors responsible for the accumulation of lipide within hepatic cells.

In most mammalian species free lipide droplets in the liver parenchyma are normally rare. However, animals such as the polar bear that have extremely fatty diets do commonly display abundant free droplets. And in most species there is a transitory deposition of lipide in the liver subsequent to a meal [54]. Our principal concern here is not with lipide of direct alimentary origin, however, but with that accumulating as the result of bodily or hepatic disturbance.

The total amount of lipide extractable from the livers of most species normally comprises between 2 and 5 per cent of the wet organ weight. At these levels, the lipides consist mostly of phosphatides and triglycerides, with some steroids [154]. When a fatty liver develops, however, the concentration of total lipide rises to 25 or even 50 per cent. This, then, is fatty infiltration, not fatty degeneration [158]. Usually the principal component of the additional lipide is triglyceride, and it is this which apparently forms the free droplets [103]. When the fatty liver develops as the result of a high-cholesterol diet, however, some of the extra lipide is steroid [84, 164].

The excess lipide deposited may be derived from dietary precursors, as when the diet is high in lipide or carbohydrate, or low in lipotropic factors (for example, choline), or it may represent lipide that has been mobilized from the adipose tissues, as when the animal is fasted [103], given growth hormone [92], or subjected to a hepato-toxin like chloroform, carbon tetrachloride, or phosphorus [102]. The use of deuterium-marked lipides and precursors established these two sorts of origin for excess hepatic lipides [13, 152].

For the histophysiologist it seems noteworthy that in most of these conditions the droplets accumulate first and most abundantly in the central zone of the liver lobule. This appears to be true for fatty livers that result from such different causes as starvation, chloroform poisoning, choline deficiency, and high-carbohydrate or high-fat diets (Figs. 85, 86) [53, 54, 96, 102]. Midzonal deposition occurs in some of these instances when the central cells have become severely necrotic. Exceptions in which the initial deposition is periportal occur with phosphorus poisoning [102, 122], and with diets deficient in certain amino acids like tryptophan or lysine [149].

The general occurrence of primary deposition of free lipide in the central zone suggests that it depends not on the initiating cause but on intrinsic properties of the liver. Since the classical studies of Noël [133a], histophysiologists have observed numerous indices of the relatively poor condition of the cells in the center of the lobule, in other words those farthest from the incoming supply of blood. It seems probable that this factor is of primary importance in the greater susceptibility of the central cells to the accumulation of lipide [53, 54, 96, 102, 167].

A key cytological index of the lower fitness of the central as opposed to the peripheral cells in the liver lobule lies in the smaller number and size of mitochondria in the central zone (Figs. 89, 90). This observation takes on added significance with the recently accumulated evidence that mitochondria contain most of the enzymes concerned with fatty-acid oxidation [145]. The relative paucity of mitochondria in the central cells would probably produce a lesser capacity to handle lipides present in the cell. In cases in which the fatty liver results from a direct or induced deficiency of choline, recent biochemical experiments [4, 5] suggest that a principal factor may be in the resultant inability of the mitochondria to maintain their normal architecture and enzymatic activity with the failure to synthesize new phosphatides.

Unless the accumulation of lipide in the hepatic cells is of long duration, the droplets are generally small and discrete (Fig. 86). With protracted insult, however, the droplets may coalesce, so that the cells acquire the appearance of white-fat cells (Fig. 87). Indeed, in prolonged choline deficiency, Hartroft [96] has demonstrated that adjacent lipide-filled cells may fuse to form giant, multinucleate structures, each containing a single, large drop of fat (fatty cysts). His evidence indicates that the development of unilocular cells, whether fused or not, precedes frank necrosis and scarring (cirrhosis). Liver necrosis, of course, does not necessarily depend on fatty infiltration [102].

Few special histochemical studies appear to have been made on lipide droplets in liver cells. Most workers have merely demonstrated their sudanophilia. In a study of the lipides that accumulated in the livers of rats fed a highly purified diet deficient in several B vitamins other than choline, Deane and McKibbin [60] reported that the sudanophilic droplets in fixed tissue behave much like those in adrenal cortical cells. That is, they were Schiff-positive (Fig. 88) and fluorescent, and contained birefringent crystals. The more recent appreciation of the first two reactions (above, p. 234) suggests that the fatty acid moieties in the droplets were quite unsaturated, a likely conclusion in view of the chemical evidence that liver lipides are generally more unsaturated than adipose-tissue lipides [22]. Dr. R. C. Goldberg (1952, unpublished results), in a study of the histochemistry of lipides present in the livers of mice injected with ethionine [111], observed that the various oxidizing agents tested by Deane and Andrews [56] on ovarian lipides all produced a detectable pseudoplasmal reaction in the droplets of unfixed liver.

Whether the occurrence of crystals in the droplets of fixed livers [60, 84, 173] bespeaks the presence of cholesterol esters or whether other lipides have crystallized during fixation remains to be determined. It would seem likely that when a fatty liver is induced by a high-cholesterol diet [84], sufficient cholesterol might occur within the droplets to be detected by the Schultz reaction.

STEROID-PRODUCING GLANDS. In most mammals the parenchymal cells of the *adrenal cortex,* some of the interstitial (Leydig) cells of the *testis,* the theca interna cells around the follicles, the lutein cells and some interstitial cells of the *ovary,* and the syncytial trophoblast of the *placenta* contain abundant lipide droplets with distinctive histochemical properties [61]. From a wealth of chemical [143] and histological evidence [61], it seems probable that these droplets represent stores of potential precursor materials that may be converted into steroid hormones when the proper stimulus occurs. To express this in more dynamic terms, lipides are probably being continually contributed to and removed from these droplets, so that their size and abundance represent a visible expression of the existing equilibrium. Most of the data about such droplets that will be given below refer to the adrenal cortex, but similar though less complete data exist for the other steroid-producing glands [22].

Using the Folch *et al.* [81] method of extracting the lipide, Karnovsky and Deane [105] found that in the adrenal gland of the rat lipides comprise about 12 per cent of the wet organ weight. The lipides contain 25 per cent cholesterol [174], or 35 to 40 per cent chromogenic steroid [109], and 30 to 35 per cent phosphatide (Tables 1–4). (Phosphatide is calculated by multiplying lipide phosphorus by 25, since phosphorus constitutes 4 per cent of the weight of lecithin.) Since as much as 80 per cent of the cholesterol in the adrenal appears to be esterified [22, 125], the amount of simple triglyceride must be relatively low, perhaps about 25 per cent of the total lipide. A certain fraction of these lipides, particularly of the phosphatides and steroids, occurs in lipoprotein structures in both the cortex and medulla, but most of them appear to be aggregated in the lipide droplets of the cortical cells.

Histochemically [61, 105], the droplets in steroid-producing cells are strongly sudanophilic (Fig. 91) and contain detectable quantities of cholesterol by the Schultz test (Figs. 72, 92). Some of them also appear to contain phosphatide, as detected by the Baker method. In addition, they present a strong pseudoplasmal reaction (Figs. 64, 84), a corollary of the fact that the fatty-acid moieties, whether esterified with glycerol, glycerophosphate, or sterol, are remarkably unsaturated (Table 2).

Within the individual gland and indeed within individual cells, not all droplets react to this entire battery of tests, a point first stressed by Feldman [78]. Cholesterol and its esters (Schultz reaction and birefringent crystals) tend to occur in the larger droplets (Fig. 92), whereas the pseudoplasmal reaction is more intense in the smaller droplets. These distinctions between droplets of different size become more conspicuous under conditions in which the cells are subjected to increased or reduced stimulus. As an example, let us review the changes occurring in the cells of the zona fasciculata, which appears to be entirely under the control of pituitary corticotrophin. When circulating corticotrophin rises, either during physiological stress or when exogenously administered, the cells of the fasciculata enlarge, the droplets become smaller (though often more abundant), the cholesterol reactivity of the droplets frequently declines (Figs. 72, 73; Table 3), but the carbonyl intensity remains high as normal. Contrariwise, if corticotrophin is cut off, either by hypophysectomy or by the administration of glucocorticoids, the cells of the fasciculata shrink conspicuously, their droplets coalesce, the cholesterol reaction may remain intense, but the carbonyl reactivity slowly declines (Figs. 94–97). In the latter situation, chemical data (Table 4) reinforce the histochemical data in indicating that inactivity is accompanied by a steady decline in highly unsaturated fatty acids and by an initial rise in the concentration of cholesterol [110, 144].

TABLE 4. Average Data for the Adrenal Lipides from 20 Normal Female Rats, from 20 Female Rats Hypophysectomized for 3 Weeks and from 20 Female Rats Hypophysectomized for 6 Weeks. All Animals Maintained on Purina Laboratory Chow and 5 Per Cent Glucose Water [105]

Rats	*Mean Gland wt., mg.*	*Lipide in Gland, %*	*Iodine Value*	*Titre of Polyene Fatty Acids **	*Cholesterol in Lipide, %*
Normal	31.9	14.3	105.2	163	25.6
Hypox.	9.3	19.5	91.4	134	27.7
3 wks.	(−71%) **	(+36%)	(−13%)	(−18%)	(+10%)
Hypox.	7.3	14.9	90.6	114	19.4
6 wks.	(−77%)	(+4%)	(−15%)	(−30%)	(−24%)

* $E^{1\%}_{1\,\text{cm.}}$ at 233 mμ after conjugation of the double bonds with alkali.

** Figures in parentheses give per cent change from control values.

It is important to emphasize that the histochemical and chemical alterations described here do not occur acutely but may require a considerable period to become evident. For example, when the stimulus is removed, it may be several weeks before the carbonyl reactivity is detectably reduced (Figs. 95, 97; see also the corpus luteum [55, 57]). Thus to assess cell activity, primary emphasis must be placed on cell and droplet size, only secondary emphasis on droplet reactivity.

I wish to discuss one last problem, namely that free lipide droplets are not invariably present in presumably steroid-producing glands. The corpus luteum frequently fails to display visible droplets when it is apparently secreting most rapidly [55, 57, 74]. More striking, perhaps, is the fact that in some species the adrenal zona fasciculata normally lacks detectable droplets. Thus, whereas the adrenals of primates, carnivores, swine, horses, and most rodents contain an abundance of free lipide droplets throughout the cortex (Fig. 91), the cortex of cattle, sheep, and those rodents capable of hibernating contains almost none [24, 133, 162] (Fig. 93). Two possible explanations for this latter condition may be offered. On the one hand, these animals may utilize much smaller quantities of adrenal hormones than most mammals and hence may not store significant quantities of precursors. This possibility that adrenal hormones may not be required for resistance to stress is suggested by the fact that the hamster, for example, in comparison to other rodents, fails to evince marked hypertrophy of the adrenal cortex when exposed to physiological stress [59]. On the other hand, Verne and Hébert [162] have suggested that ruminants and mammals that hibernate may have so evolved that hormone synthesis occurs directly from precursors derived from the blood rather than from ones that have been stored intracellularly. Whatever the explanation, it must be concluded that steroid-hormone synthesis need not always be accompanied by the accumulation of visible lipide droplets. Such a condition poses considerable difficulty for the histophysiologist.

In closing this section, brief mention should be made of some of the enzymes that characterize steroid-producing glands. For example, Zweifach *et al.* [176] have observed that dehydrogenases are normally abundant in the cortical cells, but such enzymes decline when there are signs that secretory activity is reduced. Dempsey *et al.* [63] noted that alkaline phosphatase is abundant in the sinusoids abutting on the active cells in the several steroid-producing glands but disappears when the cells become inactive.

4. *Summary*

The following topics have been reviewed: (a) the nature of lipide-containing structures in mammalian cells; and (b) methods for demonstrating lipides histologically, including consideration of the effects of fixation, the significance of sudanophilia, and methods for demonstrating phosphatides, steroids, and lipide-containing pigments. In the final section the histochemical properties and biological significance of free lipide droplets in mammalian adipose tissue, hepatic parenchyma, and the steroid-producing glands were considered.

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Addendum

Several pertinent articles that have appeared since this paper was submitted deserve mention. Debuch [179] reviewed the chemistry of the various classes of lipides, especially phosphatides. Lennert [182] evaluated a number of the available histochemical methods, including a number not mentioned here. Belt and Hayes [177] introduced a variant of the method for demonstrating unsaturated lipides, in which autoxidation was stimulated by ultraviolet light; the resultant aldehydes were then stained with the Schiff reagent. As is done here (see also [178]), they suggested parallelism between the intensity of reactivity under standard conditions and the iodine value of lipides. Since this paper was prepared, appreciation of the role of unsaturated lipides in essential metabolic processes has mushroomed [181]. Feigin [180] developed an improvement in the Schultz cholesterol method, whereby free cholesterol and cholesterol esters may be distinguished by the use of digitonin. Finally, Lillie [183] has further analyzed the Nile blue sulfate staining of acidic lipides such as occur in lipide pigments.

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9. *Some Aspects of Protein Histochemistry, with Special Reference to Protein Hormones*[1]

BY RUSSELL J. BARRNETT

THE FACTS THAT proteins are ubiquitous and that the separate identification of them has been evasive have not decreased the interest of biologists in these unique substances. Proteins are present in all cells and are indispensably involved in the biological phenomena of growth, reproduction, muscle contraction, and enzyme and hormone activity, to name a few. The proteins form a very diverse group of similar compounds of extraordinary complexity, with widely differing compositions and properties. They are both constituents and products of cells and occur in complex mixtures. Without doubt, the form and relationships of these proteins within a cellular or tissue environment are important to the understanding of function. Thus it becomes important to be able to identify and localize certain proteins histochemically and to relate them to both structure and function.

Luckily, proteins occur as macromolecules. To characterize each of these is virtually impossible. To investigate the structure, composition, and properties of the protein molecule requires substances that are pure and homogeneous, and studies of this sort are largely outside the realm of histochemistry. However, histochemists can be concerned with studies of some of the reactive chemical groups in proteins, the sites of certain specific proteins, the distribution of proteins in general within cells, the sites of synthesis or degradation of certain proteins, or the morphological evidence concerning the action of certain proteins.

For the study of biological structure and function from the point of view of chemical morphology, it is important to be able to manipulate tissues, and the proteins therein, systematically and gently so as to preserve and reveal the relationships which existed before the manipulation took place. Although this is difficult, and sometimes impossible, it is a prime requisite for most histochemical work. However, it is necessary that proteins be completely denatured or precipitated, and although it is well known that denaturation causes changes in structure and chemical properties, it goes without saying that a protein must be precipitated to be revealed histochemically (except in the rare instances when it is purposely extracted). The reagents used for precipitation must not block the reactive groups necessary to demonstrate the sites of the proteins. In addition, the denaturation or precipitation must not be reversed by other reagents used in the preparation of tissue sections or in the performance of histochemical tests.

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It is obvious from what has just been said that the techniques of histochemistry overlap those of several other disciplines. Yet it has not been completely possible in histochemistry to combine effectively the methods of physical chemistry or to adopt the quantitative aspects of many biochemical investigations and at the same time to satisfy completely the aesthetic requirements of morphology. It is possible, however, to demonstrate with certain histochemical procedures a specific protein within a cell.

The histochemistry of proteins is a large and forbidding subject. Any chapter, to be complete, should deal with the chemistry of fixation and the preparation of proteins [3, 18, 28, 67]. Besides simple proteins *per se* [25, 64], the histochemical demonstration of mucoproteins, mucins, lipoproteins [23], nucleoproteins [42, 55, 56], and the activity of enzymes [27] should be included. However, some of these subjects are covered comprehensively in recent reviews [see foregoing references and 26, 34, 46, 50, 54], and it did not seem a worth-while contribution to present a reshuffling of the content of these frequently excellent papers somewhat modified by my own repertory. Instead, some of the recent work on protein histochemistry with which I have been associated[2] will be discussed.

It is possible to demonstrate the sites both of proteins generally and of certain particular proteins by a component part of their amino-acid composition. Although most of the protein methods, such as the Millon method [63] or the diazonium methods [26], depend on one or several constituent amino acids for histochemical detection [50, 54], proteins may also be demonstrated by specific methods for end groups, such as carboxyl [11] or amino [66, 68]. I should like to discuss some methods for demonstrating protein-bound sulfhydryl and disulfide groups as a means of subsequently studying certain particular proteins.

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[2] Many of the experiments here represent collaborative work with Dr. A. M. Seligman, Dr. K-C Tsou, and Dr. A. J. Ladman, and with the technical assistance of Miss N. J. McAllaster.

1. Protein-Bound Sulfhydryl Groups

Histochemical methods for demonstrating these groups have been known since 1902 [32]. Some of the older available methods have been reviewed recently [10, 35, 54] and utilize nitroprusside [21, 30, 31] or ferricyanide [1, 24] as reagents. The many modifications of these methods that have been suggested (see reviews) indicate the shortcomings and the lack of specificity. Mercaptide-forming agents have been used [19, 20, 47] to localize sulfhydryls, and although these reagents may react with groups in proteins other than sulfhydryl, the best of these is 1-(4-chlormercuriphenylazo)-2-naphthol [20]. However, this reagent is difficult to prepare, different batches of reagent vary widely in their staining results, and the degree of specificity of reaction depends a great deal on the solvent used to dissolve the reagent in the histochemical test [35].

It is obvious from experiments with the above methods, and more so with those described below, that better specificity and color reaction may be obtained if the sulfhydryl reagent contains a chromogenic moiety and is an oxidizing agent, an alkylating agent containing a reactive halogen group, or a mercaptide-forming agent containing a heavy metal. Although these varieties of reagents are not necessarily specific for sulfhydryls, suitable modification of the reagents or of the incubation media may increase the specificity of reaction.

It is generally accepted [17, 37, 48, 51] that the most specific reaction for sulfhydryls arises from the fact that thiols and no other groups in protein are oxidized by disulfides at an alkaline pH. On this basis, a reagent, 2, 2′-dihydryoxy-6, 6′-dinaphthyl disulfide (DDD), was developed that incorporated a disulfide linkage, the specific oxidizing group, and a naphthol moiety for coupling to form an azo dye [8]. The reaction between this reagent and protein-bound sulfhydryl is reversible, is readily influenced by mass action [8], and obeys the Beer-Lambert law [22]. The reaction involves more than oxidation and reduction with the transfer of electrons, since a chromogenic moiety, naphthol, is transferred and linked to protein through a disulfide group, thus becoming part of the oxidation product. A colored azo dye is produced by coupling the naphthol moiety with a diazonium salt. With a monocoupling diazonium salt like Fast Blue RR (4 benzoylamino-2, 5-dimethoxyaniline), a red color is produced that varies in intensity with the concentration of sulfhydryls. With a dicoupling diazonium salt like Diazo Blue B (tetrazotized O-dianisidine), either a red (monocoupling) or blue (dicoupling) color is produced depending on the concentration of sulfhydryls.

To prove the specificity of this method, various tests, also applicable to other sulfhydryl methods, may be performed, and these are covered in detail elsewhere [8–10]. In brief, organic solvents will not extract the naphthol moiety of the reagent because it is attached to proteins through a disulfide link. Pretreatment of tissue with N-ethyl maleimide, a mercaptide-forming agent (p-chloromercuribenzoate), an alkalating agent (iodoacetate), or an oxidizing agent (iodine) completely blocks the reaction. Glutathione will reverse the reaction of the reagent with sulfhydryls, thus providing evidence for a disulfide link between the protein and the chromogenic moiety, and demonstrating the reversibility of the system, $2\ \mathrm{RSH} - 2e \longleftrightarrow \mathrm{R\text{-}S\text{-}S\text{-}R} + 2\mathrm{H}^+$. It is of interest that the reversal by glutathione takes place only before the production of the azo dye. Once the dye is produced by the addition of diazonium salts, sulfhydryl-containing reducing agents are ineffective.

Other naphtholic reagents have been introduced. These compounds after reaction with protein-bound sulfhydryls can also be made to couple *in situ* with a suitable diazonium salt to form a protein-bound azo dye. Production of several histochemical reagents in this manner is important. It provides the opportunity of cross-checking methods and allows the spectrum of reactions that sulfhydryls undergo chemically [17] to be made histochemically. In addition, since native proteins and enzymes usually assume a rigid steric configuration [49], the sulfhydryl groups in them may exhibit different degrees of reactivity, owing to different orientation of the sulfhydryl groups within the protein, especially in unfixed preparations. Such differences may conceivably be demonstrated by various reagents for detecting sulfhydryl groups.

Recently, N-substituted maleimides were introduced as compounds which react rapidly and specifically with sulfhydryl groups [29], and a growing use is being made of these compounds as sulfhydryl inhibitors.

Advantage was taken of these properties, a series of hydroxynaphthyl-N-maleimides was prepared, and the usefulness of these reagents for histochemistry was explored [62, 65]. Of this group, N-(4-hydroxy-1-naphthyl) maleimide was found to be best suited for histochemical procedures and compared favorably with the DDD method as to specificity and distribution of reaction in a variety of tissues [62]. N-(4-hydroxy-1-naphthyl) isomaleimide was also produced, and it likewise provided a suitable reagent for sulfhydryls. N-(4-hydroxy-1-naphthyl) maliamic acid did not react with sulfhydryls, and N-(1-naphthyl) maleimide and N-(4-acetoxy-1 naphthyl) maleimide provided new specific blocking agents [65]. Although these later compounds did not couple with diazonium salts, the latter one would couple after it had reacted with protein-bound sulfhydryls and the acetoxy group had been hydrolyzed with sodium hydroxide.

Since it is known that quinone groups react with sulfhydryls, 4-naphthoquinone monobenzenesulfonimide was produced, and tested satisfactorily [65]. In addition, a new mercaptide-forming agent, 4-hydroxy-1-naphthyl mercuric acetate, and an alkylating agent, 4-(N-iodoacetyl) amino 1-naphthol, were produced [15]. Both of these reagents reacted more slowly than the maleimides and DDD. Nonspecific staining occurred with the mercaptide-forming agent, and the alkylating agent combined with amino as well as sulfhydryl groups.

With any method for sulfhydryls care must be taken in the preparation of tissue sections so as to leave these groups free. Fixatives containing heavy metals or oxidizing agents cannot be used, and it is best that the fixative be acid to prevent air oxidation. Mixtures of alcohol and trichloracetic acid, the latter compound alone, and Carnoy's fluid are adequate fixatives from this point of view, although some proteins are soluble in them or become soluble when tissue sections are introduced into water during rehydration or during incubation in a dilute alcoholic solution containing a naphtholic reagent. Formalin can be used as a fixative, even though it reacts with sulfhydryls to form a thiazolidine carboxylic acid [60]. This reaction is easily reversible at neutrality and at room temperature [28]. However, one difficulty in demonstrating only free sulfhydryls is that there is no fixative which will retain almost all the proteins in cells and still not block the thiols. This becomes of importance, as indicated in subsequent paragraphs, especially when some proteins containing disulfides are to be demonstrated.

2. Demonstration of Sulfhydryls and Disulfides or Disulfides Alone

The reagents used to demonstrate sulfhydryls can be used to demonstrate disulfides as well after the latter groups are reduced. For a method using DDD [9], for which the other naphtholic reagents may be substituted, two parallel sections are needed. One is stained for free sulfhydryls alone and the other for sulfhydryls and disulfides after reduction of the disulfides with freshly prepared thioglycolate or other suitable reducing agents. The sites of disulfides can be inferred from the differences in staining between the two sections. Potassium cyanide may be used to split disulfides since it produces one sulfhydryl for each disulfide, and the remaining sulfur is incorporated in a thiocyanate group. The use of these as well as other reagents to produce sulfhydryls from disulfides is reported elsewhere [9, 10].

To stain disulfides alone sulfhydryls must be blocked, disulfides split, and the resulting sulfhydryls stained. This is difficult with most sulfhydryl blocking agents, since the blockade can be reversed during reduction of the disulfides with sulfhydryl-containing reducing agents. Both N-ethyl maleimide and iodoacetate, however, will block the sulfhydryls, so that the disulfides may be split with potassium cyanide [9], thereby allowing the demonstration of only disulfides. As suggested earlier, after the sulfhydryls have reacted with a reagent such as DDD and a colored azo dye produced with a diazonium salt, the naturally occurring disulfides can be reduced without affecting the quality or quantity of the azo dye that labels the sites of sulfhydryls. A second reaction can be performed in which the same or another naphthol-containing reagent is reacted with the sulfhydryls produced from the reduction of disulfides. Then the addition of a different diazonium salt will produce an azo dye of different color from the first. In this manner sulfhydryls and disulfides can be revealed by two different colors in the same tissue section [7], provided they are in different sites. When they are together, a blending of the two colors occurs.

Both sulfhydryls and disulfides may also be demonstrated with a dilute alkaline solution containing a tetrazolium salt [9, 35, 53]. This method is by far the simplest, but caution concerning the specificity of the reaction should be exercised, since colorless, soluble tetrazolium salts will be reduced to insoluble, colored formazans by groups in tissue other than sulfhydryls. Since some of these groups are water soluble (ascorbic acid, glucose) and deparaffinized sections contain few aldols or ketols, it has been assumed that sulfhydryls were the only reducing groups stained [35]. However, the simple control of iodine oxidation followed by reduction and staining with tetrazolium salts should indicate the specificity for sulfhydryls [9]. Although sulfhydryls in tissues reduce tetrazolium salts, the alkalinity necessary for this reaction (above pH 8.5) also splits the disulfides in the section. Since there is no assurance of demonstrating sulfhydryls alone and since disulfides are split slowly by dilute sodium hydroxide, it is reasonable to introduce potassium cyanide into the medium containing the reagent [9]. Cyanide does not affect the activity of the tetrazolium salt, produces one sulfhydryl from each disulfide by addition, and provides the alkalinity necessary. Of the tetrazolium salts tested, neotetrazolium was best suited for this method.

Specificity tests for the demonstration of both sulfhydryls and reduced disulfides are the same as those for sulfhydryls alone and have been referred to above, in the description of the DDD method. Fixation of tissues for both sulfhydryls and disulfides can be of wider range than those used for sulfhydryls alone [9]. Mercaptide-forming agents, e.g. sublimate-formol, or oxidizing agents—for example, Zenker's fluid—may be used, and the disulfides are unaffected by this sort of fixation. The sulfhydryls may be oxidized by the fixative to disulfides or blocked by formation of mercaptides with a heavy metal. These groups may still be revealed, since sulfhydryl-containing reducing agents will reverse the blockade of the heavy metal while reducing the disulfides produced by oxidation as well as the naturally occurring disulfides [9, 10]. These fixatives precipitate more protein than do formalin or dilute alcohol, and the precipitation is for the most part irreversible for the histological and histochemical methods subsequently used. It should be recognized, however, that, despite the effectiveness of some fixatives like Bouin's solution in retaining protein, some of the material may be lost from the tissue during preparation of tissue sections or during the reduction of disulfides. When dealing with problems concerning specific proteins in tissues that can be obtained in an isolated and purified form, *in vitro* tests should be performed to determine whether or not the protein can be retained in an insoluble state and whether its groups can be stained specifically.

3. *Distribution*

Sufhydryls are widely distributed in tissue [5]. Only collagenous fibers, the matrix of bone and tracheal hyaline cartilage, mucus and the cortex of the distal half of hair shafts, are unreactive. In contrast, all epithelia contain sulfhydryls, although the quantity varies among epithelia of different tissues. Muscle fibers, some of the elements of nervous tissue, and various blood and connective tissue elements are reactive for thiols. Figures 98 and 99 show the distribution of sulfhydryls in the cells of the liver and the kidney cortex, respectively, after fixation in alcohol-trichloracetic acid. In the former organ a homogeneous reaction is obtained throughout the lobules, and in the latter the intensity of the reaction varies with the amount of cytoplasm in the various cell types found in the kidney cortex. In some organs the staining reaction of one cell type predominates. For example, in the retina (Fig. 101) the distal segments of the rods stain more intensely than any other component, and in the testis the sperm stain most intensely (Figs. 100, 103).

When fixatives such as Zenker's fluid or sublimate-formol are used, more protein is retained, and differences in staining of different cell types or cell components are noted that are not apparent after alcohol-trichloracetic acid. Since the method for sulfhydryls and disulfides must be used after these fixatives, it cannot be estimated by these experiments alone whether the retained protein contains sulfhydryls, disulfides, or both groups. Several examples are illustrated. The brush border of the proximal convoluted tubule is intensely reactive after this procedure (cf. Figs. 99, 102), and the parietal cells of the stomach stain intensely (Fig. 107). Elastic tissue is reactive after this fixation (Figs. 106, 108) as well as globules of mucoid material in the trachea (Fig. 108). The zymogen material in the apices of pancreatic acinar cells is reactive for sulfhydryls and disulfides after Zenker fixation (Fig. 110) but not after alcohol-trichloracetic acid (Fig. 109). Several additional examples with reference to proteins of the pituitary gland will be described later. Some structures, however, contain only disulfide groups and stain intensely regardless of the type of fixative used.

Some of the keratogenous tissues provide an interesting demonstration of sulfhydryls and disulfides, since these groups are in different locations [5, 9, 12]. This is the case for the hair shaft; the proximal part, up to the region of "horny transformation," is sulfhydryl-positive (Fig. 105), the rest of the shaft is disulfide-positive (Fig. 104). However, these findings in hair, usually referred to as the prototype of hard keratin [31], are not present in other hard keratins or in soft keratin. For example, all regions of the epidermis, claw, horn, hoof, feather, and scale of various animals contain free sulfhydryls [12]. In the epidermis, disulfide groups are not restricted to the cornified layers of the stratum corneum, but are found in the stratum malpighii as well. A so-called "keratogenous zone" [31], which is rich in sulfhydryls and is supposed to occur only in hard keratin can also be found in the epidermis (Fig. 104) during a stage in the hair cycle. A transitional zone (stratum lucidum) that stains poorly with histological methods is rather characteristic of the epidermis, but such a zone does not exist in sections stained histochemically for sulfhydryls and disulfides [9]. In addition, there appears to be neither a fundamental similarity in the distribution of sulfur in all varieties of hard keratin nor a diagnostic histochemical difference between all hard and soft varieties [12]. Rather it seems that keratins are merely homologous proteins that are heterogeneous chemically.

4. Histochemical Demonstration of Protein Hormones

For the remainder of this chapter I should like to discuss some experiments concerned with the demonstration of certain protein hormones. Since some of these proteins contain large amounts of cystine, it was thought possible that they could be demonstrated by staining their disulfide groups. The success of such a demonstration depends largely on the avoidance of solution and on the extraction of a hormone such as insulin. Therefore, *in vitro* experiments were first performed, and it was found that crystalline insulin, precipitated from solution by Romeis' fluid, remained insoluble during the histological procedures necessary for the preparation of tissue sections and during the histochemical steps necessary for the specific staining sulfhydryl and disulfide groups [13]. In fact, under these conditions precipitated insulin was stained with a method for disulfides using DDD as the reagent. When the results of the *in vitro* experiments were applied to appropriately fixed sections of pancreas of various species, it was found that the beta cells of the islet of Langerhans are strongly positive for sulfhydryls and disulfides, dissolved insulin, both alpha and beta cells stained weakly and could not be differentiated on the basis of the staining reaction.

To augment the evidence provided by the *in vitro* studies and the staining of normal islets of various species in which insulin has been preserved *in situ* or extracted, additional information was obtained by physiological studies in which the quantity of insulin in the beta cells had been experimentally altered. In this circumstance the variation of the intensity of staining of the disulfides of insulin should reflect the change in insulin content from that of the normal control. Previously [13] the pancreases of rabbits were studied after the administration of insulin, diethyldithiocarbamate, alloxan, or glucose, and after starvation and partial pancreatectomy. Some additional experiments were conducted on the pancreases of obese, hyperglycemic mice.

In this paper the results of some experiments on rats are presented. Figure 111 illustrates an islet in a normal rat pancreas in which insulin has been retained and its disulfides stained, whereas Figure 109 demonstrates normal rat islets in which insulin has been extracted before staining.

Adrenalectomy (twenty-four days postoperative with no replacement therapy other than saline drinking water) caused a slight depletion of the disulfide-positive material in some of the islet beta cells (Fig. 114). Injection of cortisone into intact rats affected the islets variably, depending upon the dosage and duration of administration. At low doses (50 mg/day for 24 days), the beta cells were slightly less intensely reactive for disulfides (Fig. 113) than in the normal islets (Fig. 111). However, at higher doses (2.5 mg/day for twelve days), the islets were hypertrophied and contained no disulfide-positive material in their beta cells (Fig. 112). The latter animals, which had glycosuria, lost considerable weight and some of them did not survive the treatment. Parallel sections of pancreases stained with aldehyde fuchsin [33], a selective stain for the granules of beta cells, gave similar results, but this dye does not stain crystalline insulin *in vitro*. The results on the cortisone-treated rats with a specific staining method confirm the results of others on the effects of low [38] and high [41] doses.

Experiments similar to those described for insulin have also been conducted to demonstrate the sites of neurohypophysial hormones [6], since these also contain disulfide groups. As a result of *in vitro* experiments on the precipitation and solubility of these principles, it was found that when fixatives are used which do not precipitate the neurohypophysial hormones, only a weak staining of the axons of the hypothalamo-hypophysial fibers for sulfhydryls and disulfides occurred in the neurohypophysis (Fig. 115). However, after sublimate-formol or Zenker's fixation, which precipitated commercial neurohypophysial hormones irreversibly for the methods used, a globular or granular material was retained in the cell bodies and processes of the hypothalamo-hypophysial system of fibers which was intensely reactive for disulfides (Fig. 116). This material appeared to be morphologically indistinguishable from selectively [33] stained neurosecretory material [61], but the two had different solubility characteristics. These findings suggest that the neurosecretory material is an associated protein carrier for the active principles.

Physiological experiments on rats indicated a clear relationship between the disulfide-positive material and the hormones of the neurohypophysis [6]. It was found that the disulfide-positive material was depleted from the neurohypophysis upon dehydration (Fig. 117) and that it reaccumulated upon rehydration. The quantity of material varied with excessive hydration, and it was depleted within ten minutes after painful stress. Section of the infundibular stalk also caused disappearance of this material in the pars nervosa of the pituitary (Fig. 118), distal to the site of section, and accumulation of it in the median eminence of the hypothalamus, proximal to the section.

During the experiments just cited it was found that after sublimate-formol fixation the acidophiles of the adenohypophysis were strongly reactive for sulfhydryls and disulfides (Fig. 119), while the basophiles and chromophobes were not [39]. Since this reaction did not occur when the pituitaries were fixed in alcohol-trichloracetic acid (Fig. 115), the possibility was raised that some of the hormones in the acidophiles of the adenohypophysis were being stained by this reaction in the sublimate-fixed material but were extracted by the acid-alcohol. A series of experiments on rats was therefore undertaken to determine under what experimental conditions alterations in the staining for sulfhydryl and disulfide groups in the cells of the anterior pituitary would occur [40]. Although the basophiles were practically unreactive under normal circumstances, after castration there was a progressive increase in the staining of the granular cytoplasm for protein-bound sulfhydryls and disulfides (Fig. 122). The accumulation of reactive material after castration may be correlated with the increase in the quantity of gonadotropic hormones that occurs, since these proteins are found on analysis to contain small amounts of disulfides [45, 51]. In the normal condition, however, there must be an insufficient amount of these groups in the proteins to be revealed with any intensity.

When rats were fed a diet containing propylthiouracil 0.1 per cent, acidophiles as well as basophiles were completely devoid of disulfide-positive material (Fig. 121). Since severely hypothyroid animals have a reduced growth rate and exhibit adrenal atrophy [4], the possibility was raised that the staining of acidophiles in the control preparations might be due to the simple protein hormones, Luteotropin (LTH), Somatotropin (STH), and Adrenocorticotropin (ACTH). It was found that the pituitaries of hereditary dwarf mice contained no disulfide-positive material in their acidophiles; and in experiments reviewed in a subsequent paragraph it was found that both STH and ACTH are soluble in dilute trichloracetic acid, accounting for the absence of reactivity in the acidophiles of normal pituitaries fixed in this way (Fig. 115).

In order to obtain more information concerning the sites of ACTH in the pituitary, additional experiments were performed with the aim of altering the concentration of this hormone. Within one minute after application of an acute painful stress, which causes ACTH release, there was a depletion of the disulfide material in the acidophiles (cf. Figs. 119, 120). An hour after the stress, the amount of stainable material returned to about normal levels. Following prolonged stress (cold for fourteen days), the amount of disulfide material was increased, and after adrenalectomy, which causes an increase in the ACTH content, there was a parallel increase in the number of acidophiles and in the amount of disulfide-positive material. This increase could be largely prevented by the administration of either desoxycorticosterone acetate or cortisone.

It is difficult to correlate these results with the chemical structure of ACTH. Although it was originally found that this hormone contained over 8 per cent disulfides [43], it was subsequently shown that no sulfur was present in more recently isolated and purified samples [2, 44]. Perhaps it is significant that experiment has shown that the disulfides in the earlier samples of ACTH may be split without any loss in biological activity [43], whereas the biological activity of other hormones depends upon intact disulfides [51]. From these facts and the results of the experiments described, it seems reasonable to suggest that ACTH exists in association with a carrier protein that contains disulfide groups, and that these are some of the disulfide groups being demonstrated histochemically in the acidophiles of the adenohypophysis.

The above experiments brought into sharp focus questions concerning the sites of the anterior pituitary hormones. The quantity of results on this subject reported in the literature attests both the importance of the problem and the differences of opinion. It appears from the recent work of Purves and Greisbach [57–59], Halmi [36], Pearse [52], and others, which combined the use of histochemical stains and endocrinological experiments, that the periodic acid-Schiff (PAS) positive material present in the basophiles of the anterior pituitary are the glycoproteins, Follicle-stimulating Hormone (FSH), Luteinizing Hormone (LH), and Thyrotropic Hormone (TSH).

In order to be able to demonstrate the sites of hormones in the anterior lobe and to indicate more conclusively that the PAS-positive materials revealed in the basophiles are the glycoprotein hormones, it was reasoned that it should be possible to expose the hypophysis containing a mixture of hormones to a reagent in which some of the hormones are rendered insoluble, while others are extracted. In this manner it should be possible to extract certain proteins from a mixture of proteins in a tissue, and comparison of stained sections of the extracted tissue with sections of the same tissue in which all the protein had been precipitated would then reveal the sites of the extracted proteins. After this procedure, which reduced the number of proteins, it should also be possible to characterize the remainder better. For each extraction method the content of hormones could be accurately determined by bioassay of glands treated in the same way as those from which sections were prepared, stained, and examined microscopically.

Experiments based on the above reasoning were first performed on the glycoprotein hormones [16]. It was first determined by *in vitro* tests that all six hormones of the anterior lobe were insoluble in absolute acetone and sublimate-formol. These two fixatives formed a baseline for bioassays and staining procedures respectively. We felt that the two procedures could be equated, since there was no difference in the number of PAS-positive cells, in the intensity with which they stained in pituitaries fixed in sublimate-formal (Fig. 123), or in those fixed in absolute acetone and refixed in sublimate-formol (Fig. 125). Absolute acetone could not be used as a histological fixative, since some of the hormones are reversibly denatured and were subsequently lost when tissue sections were hydrated before staining (cf. Figs. 123, 126).

Over forty solvents were tested in various concentrations and at various pHs, but most of them were not applicable to our experiments, since they did not remove any hormone completely (though some were partially removed). Physiological saline is an example of this; it removed a fraction of the glycoproteins present and resulted in a diminution of the amount of PAS-positive material in the basophiles (Fig. 128). Dilute ammonium hydroxide (pH 8), however, removed all the hormones, as determined by bioassay, and histologically only the reticulum of the gland remained after the extraction (Fig. 127).

Two solvents, 20.5 per cent sodium sulfate in acetate buffer (pH 4.4) and half-saturated ammonium sulfate (½ SAS), removed some of the FSH as determined by bioassay. The former solvent was more effective than the later, and neither affected the content of any of the other hormones. However, these solvents were usually inadequate for histological purposes, since they were extremely hypertonic and shrank the cells so badly that it was difficult to ascertain which basophiles were deficient in staining. Occasionally an adequate block for histological study was obtained, and sections of it showed a decrease in both the number of PAS-positive cells and in the staining of some of them. This result is illustrated by a comparison of Fig. 123 (normal control) with Fig. 124 (sodium sulfate extracted).

Other solvents gave more clear-cut results. Both FSH and TSH were completely soluble in 2.5 per cent trichloracetic acid (TCA) as determined by bioassay. LH was completely insoluble, so that pituitaries fixed in this medium, refixed in sublimate-formol, and stained with the PAS method provided a histochemical method for demonstrating this hormone. When unextracted, normal pituitaries (Fig. 129) and those of castrated rats (Fig. 130) were compared with the pituitaries which were extracted (Figs. 131, 132), it was clear that the cells which contained LH were not regionally located in the pituitary, and in some instances there was evidence that LH and FSH were in the same cell. Similarly, the cells that contained FSH were not regionally located, although some of the basophiles in the sex zone appeared to contain more of this hormone than other basophiles. TSH appeared to be contained in a separate basophile which, in addition to being PAS-positive in the unextracted pituitary, also stained with aldehyde fuchsin. In the 2.5 per cent TCA-extracted glands these cells were devoid of granules and did not stain. All three glycoprotein hormones were completely soluble in 50 per cent pyridine as determined by bioassay, and glands treated with this solvent contained basophiles completely devoid of cytoplasmic granular material (Figs. 133, 134). The number of extracted cells in the gland appearing after pyridine treatment exceeded the number of basophiles; so it is possible that some poorly granulated basophiles (chromophobes) or some acidophiles were also extracted.

Turning to the simple protein hormones—ACTH, LTH, and STH—similar solvent experiments were performed to localize their sites [14], by means of the appropriate bioassays, and by staining the sections of the anterior lobe of the pituitary with acid dyes and with methods for carboxyl [11], amino [68], and disulfide groups [9] of protein. Although this investigation is in the confirmatory stage, it appears from the evidence to date that all three of these hormones are in acidophiles. Although LTH, as well as ACTH, is soluble in acid acetone, histological results are poor with this solvent and correlations cannot be drawn. The same is the case with glacial acetic acid when used as a solvent for ACTH. However, LTH is the only protein hormone of the anterior lobe of the rat that is insoluble in dilute TCA (concentrations of 1 per cent or less) as determined by bioassay. Under this condition only some acidophiles contain stainable granular cytoplasm. When the concentration of TCA was raised to 2 per cent, ACTH was completely precipitated, and more acidophiles stained with greater intensity. At a concentration of 2 per cent TCA some LH is precipitated. Histologically, basophiles are well enough preserved by this treatment so that some of them can be recognized by their shape and distribution in the gland, and these cells do not appear to contain any granular material that is not PAS-positive and still stains with the protein methods. ACTH is also completely soluble in 75 per cent acetone and 70 per cent alcohol, whereas STH is completely insoluble and LTH partly soluble. The histological picture obtained after these solvents is somewhat confusing as concerns ACTH localization and cannot be compared easily with that seen with 2 per cent TCA, since one treatment is subtractive and the other additive.

When the amount of TCA is increased to 4 per cent, STH is precipitated as determined by bioassay. At this concentration the number of acidophiles is greater than that seen at lower concentrations of TCA, and they stain with greater intensity. The acidophiles in these preparations appear the same as those which were unextracted in the control glands.

These experiments, which combine studies on the solubilities of proteins with histochemical staining and bioassay, might also profitably be extended to demonstrating the hormones of the intermediate lobe of the hypophysis, the placenta, and the parathyroid gland.

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10. A Small Particulate Component of the Cytoplasm

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THE PARTICLES described in this chapter [1] seem to be, at the present moment, the smallest among the newly discovered cytoplasmic components of the animal cell. It appears that a cell component could not have a more attractive attribute in these years of electron microscopic exploration of the cell, and it is therefore not surprising to find that these new particles have already attracted the attention of many electron microscopists interested primarily in the fine structure of the cytoplasm. I believe, however, that the particles in question may be of wider import, and for this reason I shall try to summarize in this presentation the information we have succeeded in obtaining about them. Sometime in the future they may become of direct interest to people using other approaches than electron microscopy, in their studies of the organization of living matter.

The first part of this chapter deals with the morphology of the particles, their relation to other cell components, and their distribution in a broad spectrum of various cell types of mammalian and avian origin. The corresponding information has been gathered by me and has been used as basis for a hypothesis concerning the chemical composition of the new component and its relationship to the microsome fraction [7] of the cytochemists. The hypothesis assumes that the particles consist mainly of ribonucleoprotein and account for the ribonucleic acid (RNA) found in the microsomes. The second part summarizes the results of a combined morphological and biochemical analysis of the microsome fraction carried out in collaboration with Dr. Philip Siekevitz. The analysis was actually an attempt to verify the preceding hypothesis and to correlate, at the corresponding dimensional level, structural details with chemical composition, a prerequisite for the old desideratum of correlating structure with function.

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[1] The text of this chapter has not been significantly altered since February 1955, the date of its presentation as a lecture. The list of references, however, has been brought up to date and includes, therefore, more recently published papers.

MATERIALS AND METHODS. Most of the material examined was prepared by techniques currently used for tissue specimens in electron microscopy, i.e. it was fixed in OsO_4 solutions buffered at a slightly alkaline pH [23], embedded in *n*-butyl methacrylate [22], cut in sections ~200 to 400 A thick [37], and examined in the electron microscope (RCA, model EMU 2b) without removing the embedding plastic.

Other preparatory procedures, used less frequently, will be mentioned at the appropriate place during the presentation.

1. Morphology of the Particulate Component

In specimens thus prepared, the particles in which we are interested appear as more or less circular spots of characteristically high density (Figs. 135, 143). Most of them measure 100 to 150 A in diameter; smaller elements (80 to 100 A) are rarely encountered,[2] while larger ones (150 to 300 A) are more frequent. The diameters being, as a rule, smaller than the thickness of the finest sections now obtainable (~200 A), it follows that electron micrographs of sectioned material give a good amount of information about the tridimensional form of the particles: the circular spots represent orthogonal projections of more or less spherical or globular bodies included in the thickness of the sections. In this case, we can dispense therefore with the serial sections, reconstructions, and tridimensional models needed for understanding the form of larger and more complex cytoplasmic components such as the mitochondria or the endoplasmic reticulum. It should be pointed out, however, that in addition to the common spherical particles, rodlike particles and particles with a polygonal outline are more or less frequently encountered. It can be concluded that we are dealing with a population of small bodies in which most elements have, grossly speaking, a spherical form. Considered in detail, the shape of these bodies may prove to be more complex. The polygonal or angular outlines suggest that at least some of these particles are bodies limited by many plane facets.

Most of these particles measure ~150 A in diameter and thus fall in the dimensional range usually ascribed to macromolecules. For a better orientation in terms of molecular dimensions, it may be remembered that a molecule of edestine, a protein with a molecular weight of 300,000, has a diameter of 80 A according to measurements made on electron micrographs [13], while a particle, or macromolecule, of bushy stunt virus has a molecular weight that rises into millions (9.4×10^6) for a diameter of 150 A [46].

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[2] Some of them at least represent lateral sections through particles 150 to 200 A in diameter.

Finally the small particles described are characterized by what is frequently and misleadingly called high "electron density." Actually they scatter most of the incident electrons, a property which is in general conditioned by two factors, namely the concentration of solid matter and of heavy atoms in the objects observed. This low transmission or opacity for electrons may reflect the native condition of the particles, or may represent a property acquired during and through preparation procedures. As already mentioned, most of the material used in this study has been fixed in OsO_4, a reagent which, because of the high atomic weight of osmium, is frequently suspected of "staining," i.e. opacifying various structures in electron microscopy. When an appropriate organ, such as the pancreas, is fixed in formaldehyde (4 to 10 per cent) buffered at a comparable pH, the general structure of the cells appears unsatisfactorily preserved (as evidenced primarily by poor fixation of membranous structures), but the dense particles can still be recognized (Fig. 147) because they have the same size, shape, and general intercellular distribution as in OsO_4-fixed specimens. Consequently, osmium staining can be excluded as a factor determining the density of the particles, and it can be assumed that this property reflects, to a large extent, the original condition of the material.

In most cases the particles appear as dense, homogeneous bodies deserving the descriptive name of small granules, but in some instances there are indications of a more detailed organization in the form of a denser central body, or of a denser peripheral shell. Such appearances are relatively frequent in striated muscle fibers in which, as a rule, the granules are larger in size, their average diameter being ~200 to 250 A.

2. *Distribution of the Particulate Component in Various Cell Types*

Particles having the morphological characteristics described were encountered in the cytoplasm of all cell types thus far examined, with the exception of the adult erythrocyte. Our survey covered more than 40 different cell types of mammalian (rat, guinea pig, cat) and avian (chicken, pigeon) origin, but scattered reports have mentioned the presence of similar granules in algae [38], protozoa [39], and invertebrate material. It appears, therefore, that we are dealing with a cytoplasmic component of widespread occurrence, indeed so widely spread as to lead a biologist into a very familiar kind of temptation: to generalize. We may assume, therefore, until we have proof to the contrary, that the small granules represent a regular component of animal cytoplasm.

Our survey showed in addition that the particles vary considerably in number and disposition from one cell type to another. These variations are not haphazard; on the contrary, they display a consistent pattern. There are, for instance, cell types which regularly possess a large population of granules, as well as those which, with equal regularity, contain only a few. Not only does the number of particles vary in a characteristic way with the cell type, but also their relationship to other cell components varies, especially to the endoplasmic reticulum, the system of membrane-bound cavities recently described in the cytoplasm of animal cells [35, 28]. The characteristic variations evidenced by the survey mentioned are illustrated by the following series of examples which starts with exocrine glandular cells, because it was in such cells that the particles were first seen. As is well known, the exocrine or acinar cells of the pancreas, as well as the acinar cells of salivary glands, are cells of special interest for cytologists and cytochemists because of the remarkable affinity for basic dyes shown by the cytoplasm of the basal half of their cell bodies. In addition, the same cytoplasm displays, after fixation by acid- or alcohol-containing fixatives, a laminated structure disposed either in palisades or in tight whorls called *Nebenkerne*. This type of basophil cytoplasm was described and studied in detail toward the end of the last century by a group of French cytologists from Nancy. One of them, Charles Garnier, impressed by its abundance in glandular cells, arrived at the conclusion that this was a type of highly active cytoplasm [10, 11] and tried to find an appropriate name for it. He chose "ergastoplasm" [10], a word which could be translated as "productive cytoplasm" or in more modern terms "actively synthesizing cytoplasm." Garnier's discovery and hypothesis gained in stature when Brachet [2] demonstrated that the substance mainly responsible for cytoplasmic basophilia was ribonucleic acid, a compound thought to be linked to the process of protein synthesis [5, 3] in an obligatory though poorly understood way.

When typical ergastoplasm was examined in the electron microscope (Fig. 135), it became apparent that this type of cytoplasm was characterized by the presence of a well-developed endoplasmic reticulum showing a noticeable degree of preferred orientation. In sections, the profiles of the elements composing the reticulum appear disposed in parallel rows at more or less regular spacings. In three dimensions they correspond to vesicles, tubules, and cisternae [3] which form a succession of parallel reticular sheets interconnected by relatively frequent branchings and anastomoses [30]. Even at low magnifications it is apparent that the outer surface of the membrane limiting the various elements of the reticulum is covered by numerous granules. At higher magnification (Fig. 143) these granules appear clearly with the morphological features described: they are small, dense, and more or less spherical. Apart from these "attached" particles, relatively few granules of similar size and density appear freely scattered in the cytoplasmic matrix. A situation similar to that described in the exocrine cells of the pancreas is encountered in other acinar cells, such as those of the salivary and mammary glands.

It appears, therefore, that there are two structurally different components in the ergastoplasm of acinar cells, namely a membranous component, the endoplasmic reticulum, and a particulate component represented by the small particles described. The immediate question is which of them is responsible for cytoplasmic basophilia, or, in other words, which of them contains most of the ribonucleic acid of the cytoplasm. It is obvious that the microscopical study of acinar cells cannot provide an answer to this question, because in their cytoplasm the two components are evenly distributed and occur in close association throughout the basophil regions of the cell body. The same situation prevails in other cell types known for the intense basophilia of their cytoplasm, such as the glandular cells of the prostate, the zymogenic cells of the gastric glands, the goblet cells of the intestinal mucosa, and, finally, the plasma cells of the blood and the lymphatic tissue. In all these cells the cytoplasm, in its entirety or over large portions of the cell body, is intensely basophil, and wherever basophil material is present, the electron microscope shows large concentrations of endoplasmic reticulum with numerous associated particles.

A slightly different situation is found in the perikarya of neurons in which the basophil material is distributed in discontinuous masses known as Nissl bodies. The electron microscope shows that in such cells the endoplasmic reticulum is equally concentrated in apparently discontinuous masses in which it occurs, as in the previous examples, in association with numerous small, dense granules [31]. Within a Nissl body the elements of the endoplasmic reticulum are frequently of the cisternal type and show varied degrees of preferred orientation. As in the acinar cells, dense particles cover the outer surface of the membrane limiting the cavities of the network, but small granules of similar appearance occur in greater numbers, scattered individually or in clusters, in the intervening cytoplasmic matrix. A comparable arrangement is found in the parenchymal cells of the liver (Fig. 136) [9, 29], except that in this case the free particles appear to be less numerous than in perikarya. In these two cell types there is again strict parallelism between the distribution of cytoplasmic basophilia and the distribution of these masses, within which the endoplasmic reticulum is regularly associated with small dense particles. A suggestive detail of cytoplasmic organization is already encountered in these cells characterized by a discontinuous distribution of basophil material. Elements of the endoplasmic reticulum are present not only within the masses described but also in between them. In the second location, however, they are as a rule free of attached or associated particles [29].

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[3] A cisterna is a particular type of flattened vesicle which may assume considerable width but in general remains very shallow [28].

A different type of distribution occurs in many embryonic cells and in partially differentiated cells in adult animals. Figure 138 illustrates, for instance, the disposition found in the endothelial cells of blood capillaries in the myocardium of rabbit embryos. A number of elongated profiles belonging to the endoplasmic reticulum can be recognized in the cytoplasm, and, as in the preceding examples, small granules can be seen concentrated in large numbers on the surface of their limiting membrane. In addition to these attached particles, numerous small granules of similar size and density are freely scattered not only in the immediate vicinity of the endoplasmic reticulum, but throughout the cytoplasmic matrix. A comparable situation is found in the partially differentiated cells of the bone marrow (myelocytes), of the intestinal epithelium, and of the epidermis [4] (stratum spinosum). The cells of these various types are characterized by the diffuse basophilia of their cytoplasm. Their endoplasmic reticulum is less voluminous than in the preceding examples, and the profiles of its composing vesicles, tubules, and cisternae are scattered at random quite far apart from one another. The small particles, however, are diffusely and profusely distributed throughout the entire cytoplasm. Such a disposition may suggest that the basophilia is associated with the particles rather than with the endoplasmic reticulum, but it is readily admitted that the suggestion is not particularly strong. The two components still occur together, and one may doubt whether in the light microscope, in which basophilia is observed after appropriate staining, one can distinguish between the very diffuse distribution of one component and the less diffuse disposition of the other. This inconclusive example has, however, a merit: it indicates that the fine structure of basophil cytoplasm is different and simpler in cells that went through a shorter and less elaborate process of differentiation than glandular cells or nerve cells, for instance.

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[4] In this latter case, the elements of the endoplasmic reticulum and the small particles are restricted to islands left among developing bundles of fine cytoplasmic fibrils. These islands decrease in size as the keratinization process progresses.

As far as our problem is concerned, structural differences may become more significant in the basophil cytoplasm of very young cells just at the beginning of their differentiation process. Such cells can be found in the adult organism in many places, particularly in rapidly exfoliating epithelia. It has been estimated, for instance, that the rate of renewal of the intestinal epithelium is less than three days [21]. To keep pace with the extensive exfoliation, cells in specialized regions of the mucosa, called intestinal crypts, are dividing at a rapid rate. What is important for our case is the fact that these cells are both intensely basophil and, for all practical purposes, undifferentiated endodermal cells. While they are pushed toward the surface of the mucosa by waves of younger cells, they differentiate rapidly either into usual intestinal epithelial cells or into mucus-producing cells. In the intestinal crypts, therefore, we find concentrated within a limited space the entire process of differentiation of the intestinal epithelium. Examination of these specialized regions reveals that the cells in the zone of rapid cellular proliferation contain numerous free particles evenly and randomly distributed throughout the cytoplasmic matrix [24]. The endoplasmic reticulum is represented by only a few vesicles and tubules, many of them free of attached particles, and relatively large expanses of the cytoplasm contain no elements of the network. In the zone of cellular differentiation, the elements of the endoplasmic reticulum increase rapidly in number, more particles appear attached to their limiting membrane, while the concentration of free particles decreases. In fully differentiated, simple epithelial cells the endoplasmic reticulum is relatively well developed, the small particles are noticeably less numerous than in the undifferentiated or partly differentiated precursors, and most of the particles present are attached to the limiting membrane of the reticulum.

We find, therefore, in the epithelium of intestinal crypts cells known for the intense basophilia of their cytoplasm which differ noticeably in their cytoplasmic organization from acinar cells and from the other cell types thus far described. Their cytoplasm has a poorly developed endoplasmic reticulum but contains a remarkable amount of small, dense particles. Moreover, this is not an isolated example, for a similar situation is encountered in the germinal layer of the epidermis and in that of the oral and lingual mucosae (Fig. 139). As is well known, the germinal layer performs in these epithelia the same service the crypts perform in the intestine. A similar scarcity of elements of the endoplasmic reticulum and a comparable abundance of small particles prevail in the basophil cytoplasm of young lymphocytes in the spleen and lymph nodes. The discrepancy described becomes extreme in erythroblasts, the precursors of red blood cells. In the light of these last examples, the small particles move forward as the most likely candidate for basophilia. Their case becomes even stronger when cells known to possess an acidophil cytoplasm are examined. In some of these cells, e.g. in the spermatocytes and spermatids of seminal epithelium, the endoplasmic reticulum is well developed [25]; in others, e.g. in the mature granulocytes of the blood and the centro-acinar cells of the pancreas, the vesicles and tubules belonging to this system are less numerous. What these cell types have in common is the small size of their populations of dense particles. Indeed the membrane limiting their endoplasmic reticulum is, to a very large extent, free of attached particles, and their cytoplasmic matrix contains only a few freely scattered small granules. The difference in particle content between acidophil and basophil cytoplasm is nowhere more convincingly illustrated than in the pancreas, where cells belonging to the two categories (centro-acinar and acinar cells) can be seen side by side in practically every acinus (Fig. 140). It should be stressed that acidophil cytoplasm in general appears to be characterized by scarcity, rather than absence, of small dense particles, a finding in keeping with the known presence of RNA in all types of cells, seminal epithelia and leucocytes included [20]. Thus far a single cell type, the mature erythrocyte, has been found to contain no particles. As is known, the acidophil cytoplasm of the erythrocytes is also deprived of RNA.

Within the series of cells examined, a rough parallelism seems to obtain between known amounts of RNA [20] and estimated populations of small particles.[5] Moreover, the decrease in basophilia which in many cases accompanies cell differentiation and cell maturation is also paralleled by a decrease in the particle content of the cytoplasm as clearly illustrated by Figs. 138 and 137, which represent respectively embryonic and adult endothelial cells in the blood capillaries of the myocardium.

3. Hypothesis Concerning the Chemical Nature of the Particles

It appears from the preceding observations that out of the two components we originally found associated within the ergastoplasm of glandular cells, the particulate component is the one that occurs regularly in all basophil cytoplasm, in amounts roughly corresponding to the intensity of the basophilia and to the quantities of RNA present. The other, i.e. the membranous component, cannot be satisfactorily correlated with the affinity of the cytoplasm for basic dyes. It is poorly developed in many intensely basophil cells, and it is present in appreciable amounts in acidophil cytoplasms.

On the basis of these findings, it was postulated that the small particles described are the structural elements responsible for basophilia or, in other words, the structural elements containing most of the cytoplasmic RNA. As already mentioned this hypothesis was found in good agreement with the actual situation encountered in all cell types thus far examined.

In the light of the electron microscope findings, the classical ergastoplasm could be redefined as a close association of the particulate component with a well developed and preferentially oriented endoplasmic reticulum. In this association, and according to the hypothesis advanced, the small particles are responsible for basophilia, whereas the preferred orientation of the endoplasmic reticulum accounts for the laminated or whorl-like appearances noted many years ago by Garnier and other microscopists [19].

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[5] Quantitative data on particle populations are lacking. The latter are only roughly estimated.

4. Hypothesis Concerning the Relations between the Small Particulate Component and the Microsomes

In addition to their bearing on the morphological interpretation of ergastoplasm, our observations can help in solving certain cytological problems which have recently stirred more interest than Garnier's old findings. As is well known, twelve years ago Albert Claude succeeded in isolating, by centrifuging differentially liver homogenates, a class of particles smaller than mitochondria [7]. In addition he found that these particles, which he called "microsomes" [6], were characterized by a relatively high content of RNA, a finding that put Claude's discovery in the limelight from the very beginning. The microsomes were found at a time when, for good reasons, general interest in nucleic acids was at a high level. More recent work has shown that the microsomes are distinguished from other cell fractions by the highest rate of incorporation of labeled amino acids into proteins, both *in vivo* and *in vitro* [1, 4, 17, 18, 40, 47]. In spite of all these remarkable properties, however, the microsomes have remained for many years a cytochemical concept only, without any known morphological counterpart in the organization of the intact cell. Some investigators have assumed that the microsomes are actually artifacts of preparation, e.g. fragmented mitochondria [12]; others have postulated that they may derive from the endoplasmic reticulum [8, 16, 24, 35], but convincing evidence for one view or the other has not been available.

Microsomes are usually isolated from liver and more rarely from pancreas homogenates. In both materials the endoplasmic reticulum and the small particles described in this presentation occur in close association. Assuming that the microsomes are derived from the endoplasmic reticulum, it can be speculated that their properties represent the cumulative properties of two components: the small dense particles, on one side, and the limiting membrane of the endoplasmic reticulum, on the other. Here again the presence of particles might explain the high relative RNA content of the microsomes, while the membrane may account for some of the other constituents, such as phospholipides and proteins. It is obvious that an electron microscope study of isolated microsomes can help in verifying the assumption that the microsomes derive from the endoplasmic reticulum. In addition, if a reasonably "pure" fraction of small particles could be obtained from homogenates or microsomes, the main hypothesis concerning the high RNA content of these particles could be put to a direct test.

5. Relations between the Small Particles and the Endoplasmic Reticulum

At this point in the development of our problem, the association of the small particles with the endoplasmic reticulum acquires particular importance, so that it may be worth while inquiring in more detail into this subject before going further. A careful examination of the situation found in the cell types studied indicates that the small granules have particular affinity for the membrane of the endoplasmic reticulum. Indeed, they are found in close contact with the membrane surface that faces the cytoplasmic matrix, as if "attached" to this surface. As a result many of the vesicular, tubular, and cisternal elements of this system appear rough surfaced. No other membranous structure present in the cytoplasm displays a similar affinity for the granules. For example, the inside surface of the cell membrane is always free of them, and so is the limiting membrane of mitochondria and the piles of cisternae and vesicles usually found in the centrosphere region of the cell. Even a part of the endoplasmic reticulum, whose extent varies characteristically with the cell type [26], is free of attached particles and consequently appears smooth-surfaced. The only exception is represented by the membrane which limits the cytoplasm toward the nucleus and which, in many cell types, is studded with granules exactly like the membrane of the endoplasmic reticulum. It should be pointed out, however, that a number of recent observations [25, 45] indicate that the nuclear membranes are actually a continuation of the membrane limiting the endoplasmic reticulum. As such, their similar properties are not surprising. It appears, therefore, that the affinity shown by the small granules for the membrane of the endoplasmic reticulum has a certain amount of specificity. It is clearly restricted to part of this system and does not extend to other membranous structures of the cytoplasm.

The close contact of the granules with the surface of the membrane limiting the endoplasmic reticulum appears clearly wherever the membrane is perpendicularly sectioned. In such cases the granules are occasionally arranged in rows at regular intervals (Fig. 142), but such instances are rare and the general impression is one of more or less random distribution. Of course, one would like to have a full-faced view of the membrane surface before deciding how the attached granules are actually disposed thereon and how numerous they are. Such full-faced views of the membrane of the endoplasmic reticulum are available, at least over small areas, wherever a section happens to cut very obliquely through one of those large elements of the endoplasmic reticulum described as cisternae. This point is clearly illustrated by Fig. 141, which represents a limited field in the cytoplasm of a fibroblast (rabbit myocardium). It shows a series of elongated profiles of cisternal elements cut either normally at c_1 or at increasing degrees of obliquity from c_2 to c_4. The limiting membrane of the cisternae marked c_3 and c_4 appears in full-faced view over a relatively larger area. In a number of places its attached particles are found disposed in parallel double rows. In other cell types the particles form different, easily recognizable patterns, such as circles, rosettes, spirals, and loops. The information we have about these arrangements is still limited, but certain patterns are repeatedly found, either single or in combinations, in a given cell type. For instance, rosettes and circles seem to be the predominant patterns in plasma cells [24]; spirals, loops, and double rows, in the perikarya of neurons [31]; circles and double rows in parenchymal liver cells; and double rows in fibroblasts. Dispositions approaching a close hexagonal packing are occasionally encountered in the exocrine cells of the pancreas, although in this case the particles appear to be frequently disposed at random (Fig. 143).

What could be the meaning of these patterns? Do they have anything to do with the type of cell or the type of cell products? These remain evidently questions for the future. Sometimes, while looking at these intriguing patterns, I believe that I feel very much like the French explorers who, during Napoleon's expedition to Egypt, found themselves face to face with hieroglyphs. Like some of them, I am recording the patterns, and I am waiting hopefully for a biochemical Champollion to decipher their meaning.

Irrespective of any possible functional implication, the affinity of the small particles for the membrane of the endoplasmic reticulum has considerable importance from a practical point of view. In the parenchymal cells of the liver and in the exocrine cells of the pancreas the small granules label the membrane of a large part of the endoplasmic reticulum, and consequently they could be used in tracing the fate of this cytoplasmic system during tissue homogenization and cell fractionation, assuming that the association described is strong enough to resist the drastic changes introduced by these procedures.

6. Morphological and Biochemical Analysis of the Microsomal Fraction

With these considerations in mind, an attempt was made to examine in the electron microscope microsomes isolated from liver and pancreas homogenates by the fractionation procedure of Hogeboom, *et al.* [15]. To this end microsomal pellets, cut in small fragments, were fixed *in toto* in 2 per cent OsO_4 in 0.88 M sucrose and processed thereafter through dehydration, embedding, and sectioning as small pieces of tissue. This simple technical device proved to be remarkably useful. It permitted a direct comparison of isolated microsomes with intracellular structures, since both were examined in sections. It eliminated repeated centrifugations and resuspensions (for fixation, dehydration, and impregnation) which usually result in such severe losses of material that one may wonder whether the final specimen is still representative of the original microsomal pellet. Finally, with a few precautions taken during the trimming of the pellets, the device made possible adequate sampling throughout the entire depth of the sediments.

When sections of pancreatic microsomal pellets are examined, the microsomes are found to be vesicles of various sizes limited by a thin membrane, which on its outside surface bears an easily recognizable label: the small dense particles (Fig. 144). The derivation of the microsomal vesicles from the endoplasmic reticulum appears therefore well established. Almost all of these vesicles have a continuous membrane, and many of them have a relatively dense content. These findings suggest that the reticulum is fragmented during tissue homogenization and that this fragmentation is not due to mechanical tearing, which would be expected to yield irregularly torn, emptied vesicles. What we actually find could be explained by assuming that at the beginning of the homogenization, probably when the cell membrane is ruptured, the reticulum disintegrates spontaneously into a collection of independent vesicles which are dispersed in the medium used for homogenization and isolated thereafter together in the microsome fraction. A similar disintegration of the endoplasmic reticulum has been observed in cultured cells undergoing cytolysis [35], and it is usually seen at the periphery of a tissue block in cells damaged or cut open during the excision or the trimming of the tissue.

In the case of the liver, fragments of tissue and pellets of the whole homogenate and of the microsomal fraction were examined systematically in a series of experiments. The parenchymal cells of the liver, the traditional source of microsomes, possess a well-developed endoplasmic reticulum whose rough-surfaced cisternae usually show preferred orientation (Fig. 136). In liver homogenates, fragments of various sizes of the endoplasmic reticulum are still recognizable because they still bear attached particles on their limiting membranes. As expected, the homogenate also contains nuclei, mitochondria, and smooth-surfaced vesicles of varied dimensions. The pellets of hepatic microsomes (Fig. 145) were found to contain principally rough-surfaced elements of the endoplasmic reticulum, and less numerous smooth-surfaced vesicles. Bodies of unknown chemistry and function present *in situ* in the vicinity of bile capillaries (dense peribiliary bodies) represent a minor constituent of these pellets, and damaged mitochondria occur as a relatively rare contaminant. The small dense particles which dot the surface of most microsomal vesicles indicate clearly that the latter derive from the endoplasmic reticulum. In the case of the liver, many of the fragments of the network retain the usual flattened appearance of the intracellular cisternae, when the microsomes are isolated in 0.88 M sucrose and fixed in OsO_4 in the presence of the same concentration of sucrose. They swell, however, and become relatively large vesicles when fixed in an OsO_4 solution at lower osmolar concentrations (OsO_4 in isotonic saline, acetate-veronal buffer [23], and distilled water). The findings indicate that the microsomes behave like true osmometers, and again suggest that they derive from the endoplasmic reticulum by a generalized pinching-off process rather than by mechanical tearing.

The microsome pellets studied have the usual chemical features of the microsomal fraction, as shown by the data in Table 1.

It should be pointed out that our observations on the morphology of the microsomes are not entirely novel. The microsomal fraction has already been studied in the electron microscope by Slautterback [43], who was able to recognize the two components, the vesicles and the small granules, by examining dried droplets of microsome suspensions. What is new in our work is the systematic following up of a given intracellular structure throughout the various steps of the homogenization and fractionation procedures. By using sections of embedded microsome

TABLE 1. Biochemical Composition of Microsomal and Postmicrosomal Fractions of Rat Liver

First Section. The figure for DPNH-cytochrome *c* reductase activity is an average of 5 experiments with standard deviation; all other figures are averages of 10 experiments with standard deviations.

Second Section. Comparison between liver homogenates and microsome fractions derived therefrom. The figures are averages of two experiments.

Third Section. Comparison between the usual microsomal fraction (M), two postmicrosomal fractions and the final supernatant. The figures represent a single experiment.

M = sediment obtained after centrifuging a mitochondrial supernatant for 1 hour at 105,000 g;
PM_1 = sediment obtained after centrifuging the supernatant of M for 3 hours at 105,000 g;
PM_2 = sediment obtained after centrifuging the supernatant of PM_1 for 15 hours at 105,000 g;
FS = supernatant of PM_2.

Fraction	Mg. protein N/gm.*	Mg. RNA/gm.*	μg. PLP-P/gm.*	Mg. RNA/ Mg. protein N	μg. PLP-P/ Mg. protein N	DPNH-cytochrome *c* reductase Total act.†/gm.*	Act./ mg. N
Microsomes	3.09(±0.26)	3.46(±0.31)	487(±53)	1.12	157	1140(±180)	369
Homogenate	24.4	9.25 §	1885	0.38 §	77	—	—
Microsomes	3.22	2.92	488	0.91	152	—	—
M	3.09	3.04	410	0.99	133	1130	366
PM_1	0.97	0.62	34	0.64	35	395	511
PM_2	0.92	0.36	11	0.39	12	—	—
FS	5.41	0.49	15	0.09	3	—	—

* Per gram wet weight liver pulp.

† μM cytochrome *c* reduced in 60 minutes.

§ These figures are high, since DNA also gives some color in the orcinol reaction.

Source: published in reference [29].

pellets instead of dried droplets of microsome suspensions, it is easier to recognize characteristic structural details (such as the hollow appearance of the profiles and the presence of attached granules) which indicate clearly that most hepatic and pancreatic microsomes are fragments of a particular type of endoplasmic reticulum, namely a reticulum with attached particles, the combination known to light microscopists as ergastoplasm. In view of these findings the hypothesis concerning the origin of the microsomes appears satisfactorily verified. Hence we can proceed forward in our investigation about the chemical nature of the small granules.

7. *Postmicrosomal Fractions*

It may be assumed that by further centrifugation, free or detached particles could be sedimented and thus separated from the other components of the final supernatant. Accordingly, the supernatant of the usual microsomal fraction was centrifuged for three hours at 105,000 g. The pellet thus obtained was analyzed biochemically and examined in the electron microscope. The supernatant of the first postmicrosomal fraction was recentrifuged for 15 hours in the same centrifugal field, and the resulting pellet analyzed again both biochemically and morphologically. The results are shown in Table 1. By comparison with the microsomal fraction, there is a steady decrease in sedimentable protein in the postmicrosomal fractions and an even sharper decrease in sedimentable phospholipide and RNA. As a result, the RNA/protein N ratio decreases appreciably. There is also a decrease in total diphosphopyridine (DPNH)-cytochrome *c* reductase, an enzyme shown by Hogeboom [14] to be associated with, and to a certain extent concentrated in, the microsomal fraction. The specific activity of the first postmicrosomal fraction is, however, higher. In the electron microscope the first postmicrosomal fraction appears to consist of small vesicles with very few or no attached particles. Free particles appear scattered in the pellet, but only in small numbers. The second postmicrosomal fraction is even poorer in particles. It consists of a scattering of small vesicles and particles in a matrix of amorphous material. It is evident that we were not moving in the right direction; the postmicrosomal fractions contain fewer granules and less RNA than the microsomes. The few free particles present are submerged by smooth-surfaced vesicles and by the heavy proteins of the supernatant.

8. Isolation of Small Particles from the Microsomal Fraction

Because of these unfavorable results, we turned again toward the usual microsomal fraction and tried to isolate the particles present therein either by detaching them from their supporting membranes, or by destroying these membranes. Of the various reagents thus far tested, deoxycholate gave the best results. Microsomal suspensions were treated with increasing concentrations (0.1 to 0.5 per cent) of this surface active agent and then recentrifuged for two hours at 105,000 g to permit the sedimentation of disintegration products smaller than the microsomes. The pellets thus obtained were found to contain most of the RNA (80 to 90 per cent), but little of the protein and phospholipide originally present in the microsomal fractions. Actually the amounts of protein, phospholipide, DPNH-cytochrome *c* reductase and microsomal hemochromogen [44] still sedimentable after treatment decreased rapidly with the rise in deoxycholate concentration, the corresponding losses reaching 80 to 100 per cent at 0.5 per cent deoxycholate. At this last concentration the pellets consisted mainly of ribonucleoprotein, with a RNA/protein N ratio which varied from 3 to 8 from one experiment to another. When the ratios were in the 3 to 5 range, the pellets were found to consist of a conglomeration of small, dense particles, 100 to 150 A in diameter, with occasional vesicles scattered among them (Fig. 146). When the RNA/protein N ratios were above 5, the pellets, which sometimes disintegrated during OsO_4 fixation, appeared to contain a fine fibrilar material in addition to small, dense particles. Pellets of deoxycholate-treated microsomes could also be fixed with formaldehyde (Fig. 148), and the results indicated that the high density of isolated particles, like that of intracellular particles (Fig. 147), was not primarily due to opacification by osmium oxides.

It appears, therefore, that the two components of the microsome fraction can be dissociated and that the small particulate component consists mainly of ribonucleoprotein and accounts for most of the microsomal RNA. The membranous component or, in other words, the limiting membrane of the endoplasmic reticulum seems to be associated with most of the protein and practically all of the phospholipide, hemochromogen, and DPNH-cytochrome *c* reductase of the original microsome preparation.[6]

These findings uphold the hypothesis advanced as a result of our survey of particle populations in various cell types. In liver cells the dense particles appear to be small granules of ribonucleoprotein, and as such they could be considered as the structural elements responsible for cytoplasmic basophilia. It should be pointed out, however, that the correlation in question has been established only for liver cells and that other cell types must be investigated before concluding that the small, dense particles of all cells consist of ribonucleoprotein.[7] Additional support for our hypothesis can be derived from the work of Petermann and co-workers [32–34], who succeeded in separating from liver and spleen homogenates a "macromolecular" component consisting mainly or entirely of ribonucleoprotein. In addition they were able to subfractionate these "macromolecules" into a number of categories according to their sedimentation constants and surface charges. In all probability their "macromolecules" and our small particles represent the same material.

There are numerous functional implications to be envisaged as a result of the observations and findings presented. Perhaps the most important one is that the functional unit in protein synthesis is not the "microsome" but the small particle, or a special grouping of small particles. Protein synthesis obviously proceeds in erythroblasts and in the epithelia of intestinal crypts in the presence of a poorly developed endoplasmic reticulum. The association between the particles and the limiting membrane of the reticulum usually appears in cells in which the process of protein synthesis is characterized by high output and standardized production. What could be the meaning of this association? With our findings in mind, it may be assumed that the reticulum provides appropriate surface for the arrangement of the small granules, but thus far we do not know whether the patterns described have any significant role in protein synthesis. Available information suggests, however, other possible reasons. In certain cells, for instance, the cavities of the endoplasmic reticulum appear to be in continuity, at least intermittently, with the extracellular medium through invaginations of the cell membrane [26]. In such cases the supposedly fluid phase that occupies the cavities of the network may contain a variable amount of extracellular fluid. It is possible that many raw materials, coming from outside into the cytoplasm, reach the particles through the labyrinth of the endoplasmic reticulum. In other cells the cavities of the endoplasmic reticulum appear to be used for the storage of a cell product. In plasma cells (Fig. 149), thyroid epithelia, and fibroblasts they are frequently found distended and filled with an apparently amorphous mass of appreciable density.[8] There are, in other words, numerous appearances suggesting that the cavities of the reticulum serve as feeding channels and storage space for the activity of the particles. Their association may have the same significance as the location of our plants along convenient ways of communication.

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[6] Some of these substances may represent the content of microsomal vesicles.

[7] More recently [30] both attached and free particles separted from pancreas homogenates (guinea pig) were found to consist of ribonucleoprotein. In the case of the pancreas, small particles were isolated directly from the homogenate as sizable and relatively "pure" postmicrosomal fractions.

Obviously not everything is new in this presentation. Small, dense particles have already been noticed in the cytoplasm of a few cells by Porter [36], and by Sjöstrand[9] and Rhodin [42]; "macromolecules" of ribonucleoprotein had been isolated from a few tissues, liver included, by Petermann and her co-workers, and, finally, biochemical information on microsomes is already voluminous and rapidly expanding. What is new is the bulk of the morphological information concerning the small particles and the correlated morphological and biochemical analysis of the microsomes, which lead to the identification of the small dense particles as RNA carriers. Most of the results described were obtained by using systematically the electron microscope for controlling cell fractions. The procedure may seem cumbersome and difficult; actually it became convenient and efficient because of the very simple device of fixing pellets *in toto.*

When this work was started there were some stepping stones in view in this poorly explored border region between cytology and biochemistry. Now I believe that we have a more or less continuous path. In due time it may become a good, open road.

[8] In the exocrine cells of the pancreas (guinea pig) the stored products appear as dense, well defined and relatively large bodies recently described under the name of intracisternal granules [27].

[9] More recently attached particles have been described in detail, in the exocrine cells of the pancreas (mouse), by Sjöstrand and Hanzon [41].

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11. *The Morphology of Secretion*[1]

BY SANFORD L. PALAY

TO THE CYTOLOGIST, *secretion* is the process whereby a cell elaborates, segregates, and concentrates within its protoplasm the characteristic substances emitted from the gland in which the cell occurs [106]. This definition differs from that of the physiologist, who is concerned principally with the extrusion of secretory products from the duct system of glands, and it also differs from that of the biochemist, who is concerned principally with the enzymatic chemistry and energetics involved in the synthesis of the secretory product.

Actually there is considerable overlap between the interests of all three investigators, for the cytologist is interested in the mechanism by which the secretory product leaves its cell of origin—a sort of extrusion—as well as in the mechanism by which the cellular constituents transform the raw materials reaching them from the blood stream into the final product. The special emphasis of the cytologist lies in his insistence that secretion is essentially a cellular process [107, 108] and that an adequate account of it must include the architecture of the protoplasmic framework in which it proceeds. One reason for this attitude is that in most glands the cytologist can easily demonstrate the secretory product or its precursor in the form of microscopically visible segregated granules located in the cytoplasm of the cells. These granules, their origin and fate, are what the cytologist has thought of as secretion ever since Goodsir in 1844 [45] described the preformed granules in the ink gland of the cuttlefish.

For the cytologist, then, *secretion* tends to be limited to the process of intracellular elaboration of special products in the form of granules and excludes the production of metabolites such as hydrogen ions, water, carbon dioxide, and lipides in which all cells participate. Without this restriction all cells may be considered as secretory [106] and granule-producing gland cells become merely a special case of a general phenomenon. Indeed this broader view is justified by the heuristic principle implied in all cytological investigations, that the study of particular morphological phenomena will contribute to eventual understanding of the common plan whereby the cell carries on its varied activities [1, 16]. The special case of the secretory granules, therefore, illustrates a basic cytological problem, namely the segregation of cell products from the actively synthesizing and metabolizing matrix of the cell. It is this problem which will be considered in the following discussion.

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Although little more than an epitome of the cell theory as applied to a particular biological phenomenon, the doctrine that secretion is essentially an activity of the individual cell—"the ultimate secreting structure" [45]—has developed slowly out of the efforts of nineteenth-century anatomists and physiologists to understand the secretory process. We owe particularly to R. Heidenhain [51], Nussbaum [89], Langley [65–68], Ranvier [106–108], and Altmann [1] the basic principle that there is a relationship between the intracellular secretory granules and the ferments or other specific product of glands. At the close of the eighteenth century, the secretory products of glands were believed to be circulating preformed in the blood stream, from which they were expressed into the ducts through pores of different sizes. When it became evident by the middle of the nineteenth century that glands are composed of cells, the vascular theory was displaced by the view that the secretory product was constructed in the cells of the glands and mixed with fluids derived from the blood or lymph which washed the secretory product out of the glandular walls into the ducts [51].

Although this idea is a primitive cell theory, the role of the cells in the secretory process was by no means clear. An early and persistent view was that the cell built the secretory product by transformation of its protoplasm and that when the cell was full of the secretory substance it was swept out of the gland upon a stream of lymph [51]. According to this concept, secretion was essentially a protoplasmic degeneration leading to disintegration or dissolution of the whole cell. Such ideas were particularly applied to the sebaceous glands in which the accumulation of lipide droplets was attributed to fatty degeneration [127] due to hypoxia or faulty nutrition [18 and others]. The same concept was basic to Herring's [52] interpretation of the amorphous or granular "interstitial material" in the neurohypophysis as degenerated cells derived from the *pars intermedia,* and such ideas are still being expressed [47, 48, 13]. A competing view [104] was that the cell produced secretory material which was alternately accumulated and discharged without destruction of the cell. Only late in the nineteenth century did it become clear that the production of secretory substance involved synthesis and segregation of a new material in the form of granules, and that discharge from the cell was a secondary phenomenon involving in some instances destruction of the whole cell and in others merely cyclic release of the contained product [1, 69, 107]. Nevertheless, the early theories of protoplasmic transformation are implicit in the granule doctrine proposed by Altmann [1] and further developed by M. Heidenhain [50]. According to this doctrine, secretory granules arise directly from the smallest living intracellular particles ("protomeres") by assimilation, growth, and chemical differentiation. Similarly, attempts to derive secretory granules from pre-existing intracellular organelles—the mitochondria, nucleoli, and ergastoplasm [4, 16, 54, 58, 109, 110]—which were so popular among cytologists in the first quarter of the twentieth century, echo the theory of protoplasmic transformation put forth three generations ago.

A different point of view is expressed by Bowen in his masterly review [16, p. 510]: "Secretory activity seems rather to be a function in which the whole cell-system is somehow involved, presumably in the sense that it cooperates in providing the raw or finished materials which are to be deposited in the secretory vacuoles." For Bowen, who may be taken as a representative of prevailing opinion since the 1930's, the focal point of the secretory process is the Golgi apparatus. This cytoplasmic organelle was discovered by Golgi in 1898 [43, 44] in neurons of the brain and spinal ganglia of the barn owl, dog, and cat, in pieces of tissue that had been immersed in a mixture of osmium tetroxide and rubidium bichromate for long periods of time. As originally described, this "internal reticular apparatus" (so-called to differentiate it from a superficial reticulum on the surface of neurons which Golgi described at the same time) consisted of irregular, tortuous, anastomosing threads forming a circumnuclear network which was colored yellow by reaction with the fixative. At nodal points in the reticulum were small, rounded patches with more transparent centers. Similar networks were soon discovered in virtually all cell types. Golgi himself refused to speculate on the functional significance of the reticulum he discovered, but he had no doubt that it was a real structure. The apparatus rapidly became the center of a controversy which has continued to this day.[2] The voluminous literature has been repeatedly reviewed [16, 53, 60] and will not be detailed here. Suffice it to say that the controversy has revolved about three problems: (1) the reality of the apparatus, (2) the identity of the apparatus with various formations that have been described from time to time by different methods, and (3) the function of the apparatus, particularly its relation to secretion. The first two problems are inextricably intertwined because those who state that the Golgi apparatus is an artifact almost always declare that some other organelle in the living cell is the basis for the form seen in fixed preparations.

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[2] See "A symposium on the Golgi apparatus," J. Roy. Micr. Soc., *74,* 133–240, 1955. The symposium was held on May 5, 1954.

1. The Identity of the Golgi Complex

The classical Golgi apparatus is usually demonstrated by means of the Mann-Kopsch or Kolatchev-Nassanov technique, which involves fixation of fresh tissue in Champy's fluid (a mixture of chromic acid, osmium tetroxide, and potassium dichromate), followed by treatment with osmium tetroxide for several days at 37°C. Fragments of tissue are examined each day during the post-osmication, and the reaction is considered complete when the cells display a delicate black reticulum or fenestrated platework in the cytoplasm and all other organelles are either colorless or pale yellow. Another method (DaFano) utilizes tissues fixed in a mixture of cobalt nitrate and formalin followed by impregnation with silver nitrate. As in each of these methods and their variants the reticulum is revealed by the deposition of a heavy metal in fixed tissue upon a supposedly preformed structure having a reticulate architecture, the validity of the final picture depends upon a demonstration of the corresponding structure in the living cell. Reticular clear spaces in the juxtanuclear zone have been reported and figured in the living cells of the islets of Langerhans *in situ* [90], in fibroblasts in tissue culture [74, 75], and in surviving cells of islets [6, 14], sympathetic ganglion [39], and epididymis [30, 31]. Whether these spaces represent channels (the trophospongiosum of Holmgren [56, 57] or canalicular apparatus of Bensley and O'Leary [14, 20]), or solid bars and plates in the cytoplasm is not always clear from the descriptions. According to Ludford [74], the Golgi apparatus in living fibroblasts is a "colorless reticulate structure" or a clear reticulate area in the midst of vacuoles that stain with vital dyes (in this case, methylene blue); but the clear area does not color with supravital dyes. Later, however, he [75] stated that structures resembling the Golgi apparatus are seen in living cells in tissue culture only if the cells are degenerating or are stained with methylene blue. In 1948, Ludford, Smiles, and Welch [77] published a phase-contrast photomicrograph of sarcoma cells in tissue culture, showing a finely granular cytoplasmic mass "with which there appears to be associated a canalicular or vacuolated system, suggestive of the Golgi apparatus of osmic or silver preparations." Further photographs of this type were published in 1950 [76]. The great majority of authors have not reported seeing anything resembling the Golgi apparatus in living or surviving cells [2, 4–6, 22, 26, 53, 54, 102, 124–126, 132, 133, 135].

Parat [102] was the first to assert boldly that the classical reticulum of the preparations impregnated with metals was an artifact due to the deposition of the silver or osmium upon and between vacuoles in the cytoplasm (vacuome). Covell and Scott [27] demonstrated that neutral red vacuoles in vitally stained nerve cells darkened with osmic acid vapors and were frequently aligned in strands resembling the Golgi apparatus. These observations have been commonly cited as proof that the classical reticular apparatus is simply an artifact and that its appearance in fixed and impregnated specimens gives no clue to the original state of affairs. But Beams [7] has pointed out that osmic acid does not impregnate the vacuoles of pancreatic acinar cells unless they have first been stained with neutral red, and furthermore he presented preparations in which the neutral red vacuoles and reticular Golgi apparatus were visible side by side in the same cells [7, 9; see also 30]. Therefore he drew the conclusion that the neutral red vacuoles (the vacuome) and the reticular apparatus must be two different structures.

Finally, the separation of the Golgi apparatus from the other constituents of the cell by stratification of intact cells in the ultracentrifuge [10] has been taken [60, 78] to be conclusive proof that the Golgi apparatus cannot be an artifact. This experimental evidence bolstered the conclusion that it must be a preformed intracellular organelle because of its consistent behavior under different physiological conditions, as during the secretory cycle. With such arguments the adherents of the reticular hypothesis disposed of the vacuome theory and artifact hypothesis of Parat. The suggestion that the vacuome and other substances capable of impregnation in reticular form could also move under a centrifugal field or under different physiological conditions in the cell was brushed aside [53]. By 1938 Kirkman and Severinghaus were able to declare [60, p. 418]: "Observations by experienced and competent investigators leave no doubt but that the apparatus exists and can be demonstrated in both living and fixed protoplasm."

But the ghost of Parat's vacuome was not so easily laid. Hirsch [54], in an extended series of papers summarized in his monograph of 1939, proposed a "synthetic theory" of the Golgi apparatus which brought together the vacuome and the reticular apparatus as a unified, dynamic cellular organelle. He conceived of the Golgi apparatus as arising out of a homogeneous "presubstance"—droplets, granules, or threads, sometimes organized into networks—that absorbs silver or osmium and that usually stains with neutral red. Out of this presubstance develop "Golg systems" consisting of an osmiophilic shell, the Golgi externum, and an osmiophobic core, the Golgi internum. The latter is either a granule or a vacuole, and within it the product of the Golgi apparatus forms. If the cell is secretory, the product is released from the Golgi system and extruded; otherwise, it is dissolved and utilized in the internal metabolism of the cell, while the externum reverts to the condition of the presubstance to repeat the cycle. Some of the systems may be complicated bodies having numerous internums included within one continuous externum.

Worley [131–133] described similar forms in the living and surviving cells of both vertebrates and invertebrates after staining with methylene blue. He declared unequivocally that the classical Golgi apparatus of the fixed cell is formed from chromophilic vesicles in the living cell that stain with vital dyes. Like Hirsch, he found that the Golgi system consists of a series of vesicles each with a thick pellicle corresponding to the Golgi externum and a fluid core corresponding to the Golgi internum. Shrinkage in hypertonic salt solutions resulted in collapse of the vesicles and formation of a reticulum reminiscent of the classical Golgi apparatus.

In recent years, Baker and his students [2, 4–6, 20, 22, 37, 119, 125, 126] have re-examined the whole Golgi problem with histochemical techniques. In living and surviving cells examined under the phase-contrast microscope they have never seen anything resembling a network, and they believe, therefore, that the classical reticulum is an artifact caused by deposition of silver or osmium upon lipide bodies, mitochondria, and other cytoplasmic constituents in an entirely nonspecific fashion. Baker and his colleagues have consistently found in the juxtanuclear region of living or surviving cells subspherical, colorless, refractile bodies, sometimes containing one or more vacuoles that take up neutral red. The resemblance to Hirsch's presubstance and Golgi systems with their internum and externum is fairly evident. That the bodies consist at least partly of lipide is demonstrated by the facts that they stain deeply with sudan black and impregnate with osmium tetroxide [2, 5]. The vacuoles, however, remain colorless in sudan black preparations, but with prolonged exposure to osmium tetroxide they gradually darken [125, 126]. In many cells there is also a sudanophilic and osmiophilic material diffusely spread throughout the Golgi zone [2], but Baker does not consider this diffuse lipide as a characteristic feature of the Golgi apparatus [4].

In a histochemical study of the sudanophil bodies in sympathetic neurons of the rabbit Casselman and Baker [22] showed that they consist of two components: (1) a phospholipide that is insoluble in hot or cold acetone, soluble in hot pyridine, and gives a positive reaction to the acid hematein test [3, 21] but a negative one to the periodic acid-Schiff reagent; and (2) a galactolipide (presumably cerebroside) that is insoluble in cold acetone, soluble in hot acetone and hot pyridine, and gives a positive reaction to the periodic acid-Schiff reagent but a negative one to the acid-hematein test. The corresponding bodies in the neuron of the locust [119] contain the same two components, although in apparently different proportions. The bodies in the Golgi zone of the intestinal epithelial cells of the mouse contain a sudanophilic and osmiophilic lipide but apparently no phospholipide, for the acid hematein test is negative [6]. However, corresponding structures in the neurons of the snail *Helix* [125], in the intestinal epithelial cells of the leech [20], and in human sebaceous cells [82, 85] do contain phospholipide, as shown by a positive acid hematein reaction. The cytoplasmic matrix in which these bodies are embedded also varies in chemical composition from one cell to another. For instance, in the intestinal epithelial cell of the mouse a mucopolysaccharide lies in this position, but is absent from the neurons of the locust [6]. On the basis of these findings, Baker [6] agrees with Hibbard [53, p. 14] that "efforts to generalize by assuming that the behavior, structure, and physicochemical composition observed in any one case are applicable to the 'Golgi apparatus' in the abstract sense cannot but result in contradictions. With regard to most of the generalizations, we should be better off if the discovery of the impregnation methods had never been made."

Baker and his school have introduced a new series of names for the structures they have studied. At first Baker [2] referred to the whole aggregate of juxtanuclear, lipide-containing bodies and vacuoles as the "Golgi element." Later [4], he refers to the individual lipide structures as "Golgi bodies," and Thomas [124, 125] terms the more complex, vacuolated bodies, "spheroids." In an attempt to be objective and simply descriptive, Baker [6] has recommended that the terms "Golgi apparatus," "Golgi substance," and "Golgi bodies" should all be discarded as misleading, and that the bodies usually seen in the corresponding region of living cells should be called "lipochondria," meaning simply granules or spheres containing lipide. Terms like "Golgi apparatus" only obscure our ignorance.

Baker's position may be summarized as follows. In the juxtanuclear region of the living cell there are colorless, spherical bodies, the lipochondria, which may be simple granules or vacuolated spheroid systems. The simple lipochondria and externums of the vacuoles contain a sudanophilic and osmiophilic lipide, which in only a few instances is a phospholipide. In some cells the lipochondria also contain cerebroside, whereas in others none is demonstrable. In certain cells the cytoplasmic matrix between the lipochondria contains a mucopolysaccharide, but in others this substance is absent. In agreement with Parat [102], Owens and Bensley [91], and Hibbard [53], Baker stresses the heterogeneity of the intracellular structures and substances that are blackened with osmium or silver. Therefore, he regards the impregnation techniques as "very liable to be misleading morphologically and quite useless histochemically" [6].

In 1949 Palade and Claude [95, 96] published an intensive study in which they showed that intracellular structures resembling the reticular Golgi apparatus could be produced in isolated fragments of fresh tissues and homogenates by treating them with 40–55 per cent ethanol and staining them with sudan black. Under these conditions polymorphic myelin figures developed from refringent lipide granules and droplets in the cells and duplicated the classical Golgi network. These myelin figures concentrated neutral red and their walls stained with methylene blue and sudan black. When isolated fragments of tissue were treated with the conventional Golgi fixatives, myelin figures formed in the same way and subsequently blackened with osmium to form typical Golgi nets. Treatment of homogenates with saline buffers between pH 4.8 and 6 produced similar intracellular myelin figures. Palade and Claude [96] concluded that the classical Golgi apparatus is "a gross artifact, namely a myelin figure, or a complex of myelin figures, which develop in cells during fixation, blacken during silver or osmium impregnation, and are further distorted to various degrees by later handling." The tendency of the fixatives to produce the Golgi apparatus was attributed to dissociated diffusion of hydrogen ions and electrolytes during fixation. The local acidification of the tissue induces formation of myelin figures, which are stabilized by the more slowly diffusing electrolytes and finally impregnated by reduction of osmium oxides or silver salts. Some support for this interpretation comes from the studies of liver, pancreas [135], and sarcoma cells [134], in which vitally stained lipochondria underwent myelination when the cells were treated with hypertonic solutions.

The papers of Baker and his colleagues and of Palade and Claude awakened the adherents of the classical reticular apparatus to impassioned battle. Gatenby and his co-workers [38–42] dismissed the lipochondria as being fat droplets described by Ciaccio [25] or as lipofuscin granules accumulating in the cells of older animals. They reiterated that the Golgi apparatus is visible in the living cell and consists of a canalicular system, the walls of which are osmiophilic and argentophilic and, in neurons, are associated with a sudanophilic component. According to them the intracellular distributions of the Golgi apparatus and the spheroids of Thomas or the lipochondria of Baker are entirely different. They categorically denied that the Golgi apparatus is produced by running together of osmiophilic vesicles [40]. They also rejected the view that the neutral red vacuoles could be precursors of the canalicular apparatus, as they merely are stuck on, or lie near, the classical reticulum. Gatenby and Moussa [42] disposed of the work by Palade and Claude as irrelevant "mash cytology."

In a detailed study of the Golgi problem, Lacy [61, 63] demonstrated both sudanophilic lipide bodies and colorless canals in exocrine and endocrine pancreatic tissue by Baker's sudan black method. Silver techniques (Aoyama's) impregnated the canalicular system and the lipide bodies associated with it, but not the lipide bodies in other regions of the cell. The walls of the canals did not color with sudan black unless they were first treated with silver nitrate. Lacy suggests that this characteristic indicates some type of masked lipide in the walls of the canals. He regards the conclusions of Palade and Claude as inapplicable because the canals give a negative test for phospholipide with Baker's acid hematein method and thus could not represent myelin figures derived from such substances. He concludes that the lipide bodies and the canalicular structures are two distinct entities, both of which exist in the living cell.

The discussions of the Golgi apparatus in recent years have been colored with a vehemence out of proportion to the actually determined facts [41, 42, 46, 6]. "The lack of unanimity on every aspect of the subject," writes Hibbard [53], "strongly suggests that cytologists are not all studying the same thing, in spite of the fact that they all use the same name for it." The validity of the two principal conflicting concepts cannot be decided on the basis of existing light microscopic techniques. Therefore, we must turn to electron microscopy for information that may permit interpretation of the divergent findings of light microscopy.

Dalton [28, 29] was the first to identify the Golgi apparatus in electron micrographs of tissue sections. In a study of hepatic and intestinal epithelial cells, he showed that myelin figures do not form in cells fixed by perfusion. When fragments of tissue were post-osmicated for several days, osmiophilic threads appeared in the Golgi zone in association with vacuoles arranged as the negative image of the Golgi apparatus. In a more detailed study one year later, Dalton and Felix [30] examined fresh, isolated epithelial cells of the duodenum, prostate, seminal vesicles, and epididymis and also electron micrographs of fixed tissues from the same organs, fixed in Regaud's or Champy's fluids and post-osmicated. They reported that a classical Golgi network was visible in fresh isolated cells during the first five to twenty minutes after removal from the organ when examined in the ordinary light microscope, in the phase-contrast microscope, or in dark field. When the tissues were supravitally stained with neutral red or methylene blue, the Golgi net was always refractory to staining whereas droplets in the surrounding cytoplasm were readily colored. In frozen sections stained with sudan black according to Baker's method, a network of sudanophilic strands associated with colorless vacuoles was visible in the Golgi zone. In the Champy-fixed and post-osmicated preparations examined in the electron microscope the Golgi apparatus appeared to consist of lamellated osmiophilic strands and osmiophobic vacuoles. Mitochondria were not coated with a deposit of osmium particles.

Dalton and Felix [31] then restricted their attention to the cells of the epididymis, because the Golgi apparatus here was easily visible in fresh preparations and readily photographed by phase-contrast microscopy. Their phase-contrast photomicrographs of isolated cells in hypertonic sucrose and sodium chloride show clearly a supranuclear elongated network of sinuous strands. Similar bodies were separated from the cytoplasm by homogenization and then tested with various reagents. They stained with nile blue sulfate and chrysoidin Y, but not with neutral red or methylene blue. In the intact cell none of these dyes would color the lepidosomes in the developing spermatid and of the "dispersed" Golgi apparatus of insect neurons, where the sudanophilic substance of formalin-fixed frozen sections is exactly the same in position and configuration as the osmiophilic material of classical Golgi preparations and as the packets of closely apposed cisternae in electron micrographs. The large dilatations of some of the cisternae provide a reasonable facsimile of the Golgi internum, hence the "canals" repeatedly described by the adherents of the reticular Golgi apparatus and the vacuoles of the Golgi systems described by their opponents. The association of strongly sudanophilic material in the form of caps, crescents, and rings with a colorless internum [2, 4, 37, 41, 63, 64] corresponds almost exactly with the association of dilated cisternae and the piles of flattened vesicles at their margins. Comparison of Baker's figures of lipochondria in sudan black preparations [2, 4, 5] with the electron micrographs of impregnated cells [31–33] will demonstrate the correspondence between the lipochondria in fresh and fixed preparations and the Golgi complex of electron micrographs.

The identity of the neutral red-staining vacuoles is a more vexatious problem. Bensley [14], O'Leary [70], Beams [7–9] and Dalton and Felix [30, 31] all point out that the Golgi substance does not stain with neutral red or methylene blue in surviving cells of islets or epididymis. Supravitally staining droplets, however, do occur in the Golgi region of intestinal epithelial and hepatic cells [30]. The droplets also evidently stain with sudan black and darken with osmium tetroxide. But as Gatenby [41] and Dalton [30] point out, they darken much more rapidly than does the Golgi substance itself, and Beams [7] says that they do not darken unless first stained with the supravital dye. Therefore, the neutral red vacuoles are not a consistent part of the Golgi complex. Electron micrographs of the Golgi regions of a variety of cells reveal the presence of dense droplets of either homogeneous or heterogeneous granular content, usually delimited by a dense single membrane (Fig. 167). The density of the content suggests that they have a high lipide content. It is possible but not proved that these droplets are the sudanophilic, osmiophilic droplets that stain with neutral red. A recent electron microscopic study of pancreas and kidney of mice injected with neutral red [129] showed that sudanophilic, osmiophilic bodies appear in the cytoplasm after the injection of neutral red. This study also supports the concept that the neutral red vacuoles and the Golgi complex are different cytological entities.

The foregoing discussion indicates that the Golgi apparatus is a genuine cytoplasmic organelle and that the vehemently contested divergent opinions as to its nature, constitution, and form in the living cell are not so irreconcilable as the heat of the controversy has made them appear. As Dalton [28] remarks, "Thus the great mass of literature dealing with studies on classical Golgi networks in which fixation by immersion was employed need not be discarded as representing *gross* artifact. Such studies may be rescued from limbo by considering them as dealing with a substance existent in the living cell which differs essentially from other cell components but which may, under certain conditions, be distorted to a certain extent by the classical methods used to visualize it."

2. *Secretion and the Golgi Complex*

It is a curious irony that the cytologist, so much concerned with the origin of secretory substances within cells, still finds it convenient to classify glands according to their mode of releasing the product. Thus the holocrine glands release whole cells as the secretory substance, the apocrine glands lose only the apical parts of the cells, and the merocrine glands extrude only the special product, the secretory granules. Ranvier [106], who insisted upon the cytological view of secretion, was himself responsible for this classification. The reason for this convenience is that all of the cytological theories of the secretory process have subsumed that the formation of the secretory product is in all cases uniform and that only the mode of release differs from gland to gland. Whether the theory proclaimed that glandular products were preformed in the blood and merely filtered into the ducts, were disintegrating parts of degenerating cells sloughed into the ducts, or were sequestered products washed into the ducts by streams of lymph, the distinguishing features of different glands always emphasized the mode of release from the site of origin.

A modern theory of secretion must take into account the new complexities of cytoplasmic structure that have been revealed by electron microscopic studies and the biochemical knowledge of these structures that is almost daily enlarging. Figure 167 shows a portion of the nucleus and cytoplasm of a glandular cell (in this case, an acidophile of the adenohypophysis of the rat), with all of the morphological machinery occurring in secretory cells wherever they may be located. In this figure, the nucleus with its double membrane is seen at the upper edge. The cytoplasm contains circular and elongated profiles of mitochondria, identifiable by their double membranes and transverse cristae; ergastoplasmic vesicles, consisting of closed membranes and their encrusting nucleoprotein granules; agranular endoplasmic reticulum belonging to the Golgi complex, and numerous small, round homogeneous secretory droplets. Every one of the recognized intracellular organelles has been implicated in the secretory process during some time in its history. Most of these implications deal with the origin of the definitive secretory granules in one or another organelle. The history of the controversial opinions concerning the origin of secretory granules has been admirably reviewed by Bowen [16]. Bowen felt that he had settled the problem to his satisfaction by showing that no consistent story could be constructed from the evidence except from that concerning the Golgi apparatus, in which secretory granules seemed always to appear. Yet his evidence for this conclusion was at best tentative.

Recent work with the electron microscope has given new impetus to the study of secretory cells, and the chemical characterization of most of the intracellular organelles has clarified the role of many organelles in the metabolism and synthetic activities of the cell, so that it is now possible to construct a more comprehensive theory of the origin of secretory materials, the formation of secretory droplets, and the mode of extrusion from the cell. As much of this work has been presented in previous chapters, only a brief statement of the general conclusions need be given here. Protein production in cells is correlated with the presence of basophilic cytoplasm or ergastoplasm (Fig. 161), which has proved on electron microscopic examination to consist of two structural components, a system of membrane-delimited, frequently anastomosing vacuoles, vesicles, and tubules together with a congeries of fine nucleoprotein particles encrusting the outer surfaces of the membranes [93, 94, 97]. The particles not only are responsible for the basophilia of the ergastoplasm but also evince a high and rapid uptake of radioactive amino acids, a property which indicates their active role in the synthesis of protein [72]. The function of the ergastoplasmic membranes and their contents is not yet clear, although some enzymatic activities (notably DPNH-cytochrome *c* reductase) have been localized within them [98]. As is now well known, the mitochondria are sites of oxidative activity [55, 92, 115].

Because the membranes of the Golgi complex and the secretion granules are more pertinent to this chapter, they will be discussed in more detail. As already described, the Golgi complex (Figs. 157, 163, 168) is composed of closely packed, broad, shallow cisternae and small vesicles without associated fine particulates. Like the ergastoplasm (Figs. 157, 161), however, the vesicles of the Golgi complex display frequent, rounded dilatations. The content of the cisternae and vesicles varies considerably in density. In most secreting cells, secretory droplets can be found among the elements of the Golgi complex, particularly about the periphery of the mass. In some cells—for instance, in the developing spermatid [19, 94] and in the adenohypophysis [35, 36, 111]—the Golgi complex is restricted to a condensed mass of membrane-bound vacuoles next the nucleus, whereas, in other cells—for example, in the pancreatic acinar cell [123] and in the neuron [101]—the Golgi complex is frequently dispersed in small masses or elongated chains between the nucleus and the cell surface. Usually, in secreting cells, the Golgi complex lies in the supranuclear cytoplasm, whether in a single mass or in multiple aggregates. Connections between the agranular reticulum of the Golgi complex and the granular reticulum of the ergastoplasm have been described [94, 101], and it is probable that both of these systems belong to a family of membrane-limited vacuoles that permeate the cytoplasm and are in dynamic equilibrium with one another as well as with the surface membranes of the nucleus and with the plasmalemma.

Secretory granules, wherever identified, always comprise two components: a surface membrane consisting of a thin, single layer, and an internal homogeneous substance of varied density (Figs. 157, 160, 162, 163, 167, 169, 170). In some cells the content appears to be more complex, containing membranes and droplets of heterogeneous densities. The surface membrane, however, is always smooth and in this respect it resembles that of the agranular reticulum in the Golgi complex.

A survey of typical gland cells representing the different types of glands found in mammals discloses that in each case secretory granules originate in association with the Golgi complex. The following section deals with illustrative examples from holocrine, apocrine, and merocrine glands.

THE CYTOLOGY OF SECRETION IN HOLOCRINE GLANDS. The best example of holocrine secretion is found in the cells of sebaceous glands. The glandular acini consist of large, vacuolated, central cells and smaller, undifferentiated, peripheral cells. Progressive maturity from the periphery toward the center of the acinus is indicated by the increasing accumulation of lipoid droplets in the cytoplasm until the cells are transformed into swollen sacks of lipoid which finally break down and release sebum into the lumen of the duct. The immature cells in the periphery of the acinus stain deeply with basic dyes; as this basophilia is removed by incubation with ribonuclease, it is evidently due to the presence of large amounts of ribonucleic acid [84]. These less differentiated cells also stain strongly with sudan black, although not with sudan red. The facts that their affinity for sudan black is not abolished by immersion of the sections in lipide solvents before staining and that the same cells stain intensely with Baker's acid hematein [3, 21] indicate that their sudanophilia is due to phospholipides [84]. With increasing maturity of the cells toward the center of the acinus, basophilia decreases and droplets of lipides stainable with sudan red and black and extractable with lipide solvents appear in increasing numbers. The material stainable by the acid hematein method for phospholipides becomes restricted to the interstices between droplets and to the surfaces of the droplets. The droplets of lipide increase in size until the cytoplasm is full of them. In the more mature cells the droplets contain cholesterol esters and triglycerides and very little phospholipide. The reaction to osmium tetroxide is irregular. In the peripheral cells some of the small droplets stain and others do not. In the more mature cells the droplets appear to be osmiophobe and have a shell of osmiophilic substance around them [80, 83, 84].

The Golgi apparatus of sebaceous cells has been described as a collection of curved osmiophilic bodies or batonnets, which at the onset of secretion disperse through the cytoplasm and become associated with individual small vacuoles or droplets of lipide [73]. As the droplets increase in size and number, the Golgi fragments also proliferate, so that each droplet possesses a husk of Golgi material applied at its circumference. Ludford [73] and Bowen [16] believed that such forms indicated that the secretory material of the sebaceous cell formed inside the hollow shell of the Golgi bodies. As the secretory droplets grow and fill the cytoplasm, the Golgi material becomes reduced to a thin husk and almost completely disappears in the mature cells. This osmiophilic husk about the lipide droplets corresponds in position to the material staining with acid hematein and is probably phospholipide in nature.

The mitochondria in sebaceous cells are stout rods and wavy filaments or rings and granules which have been difficult to demonstrate in stained preparations. During the formation of lipide droplets the mitochondria are scattered throughout the cytoplasm and compressed into the thin strands of cytoplasm between the growing droplets. According to Montagna [82] they do not decrease in number during sebaceous transformation and do not fragment until the cell begins to disintegrate. Contrary to the opinion of Nicolas, *et al.* [88], neither Ludford [73] nor Montagna [82] found any evidence that the mitochondria are transformed into the secretory product of the cell.

On the background of these studies let us now turn to the electron microscopy of the sebaceous cell. The cytoplasm of the undifferentiated cells at the periphery of the acini contains numerous mitochondria and only sparsely distributed vesicles, tubules, and cisternae of ergastoplasm (Figs. 150, 151). Fine granules resembling those described by Palade [93] in many cell types and identified as nucleoprotein are dispersed not only over the surfaces of the ergastoplasmic membranes but also in clusters and rosettes throughout the cytoplasmic matrix. Undoubtedly these granules represent the morphological counterpart of the basophilia so prominent in the cells of this layer. Small clusters of agranular reticulum occur in the neighborhood of the nucleus (Figs. 151, 152). They consist of more or less parallel tubular elements and vesicles in small packets. Only occasionally are droplets of lipides or structures resembling secretory granules encountered in these cells.

A remarkable change is evident in the cells of the next layer (Figs. 151–3). The agranular reticulum has increased in extent and now permeates the entire cytoplasm. The mitochondria and ergastoplasm have not changed noticeably. Numerous secretory droplets are scattered through the cell. In the early stages of their development these droplets have a peculiar structure (Figs. 152–4). As seen in the electron micrographs, they consist of a loosely and irregularly arranged network of strands (perhaps actually tubular membranes) which appear to be suspended in a clear circular or elliptical space. Delimiting this space from the rest of the cytoplasm are usually several tightly apposed layers of membranes of extraordinary architecture. Close study of these membranes in micrographs of sections cut in various planes with reference to the axes of the droplets indicates that each layer consists of an essentially bidimensional grid composed of intercommunicating tubules laid more or less at right angles to one another in the plane of the layer (Fig. 154). The tubules of the innermost grid appear to be continuous with the strands lying within the enclosed clear space, and the tubules of the outermost grid appear to be continuous with the tubules and cisternae of the agranular reticulum dispersed in the cytoplasm. The secretory droplets apparently form in the cytoplasmic spaces enclosed by the sinuous curves of the agranular reticulum (Fig. 153). As the cells mature, the cytoplasm becomes compressed into the interstices between the secretory droplets, which become larger and more numerous until only thin plates of cytoplasm containing mitochondria, fine granules, and tubules separate the droplets (Figs. 154, 155). The irregular strands within the droplets disappear and the husk of compressed membranous grids becomes thinner. The droplet now appears as a clear or empty space. Presumably the lipide which originally occupied this space has been dissolved out during the preparation of the specimen. According to the results of light microscopy (see above) it does not react with the osmium tetroxide of the fixative sufficiently to remain in place.

From the above description it seems probable that the husk of multiple agranular membranes about each droplet space corresponds to the osmiophilic shell described by Ludford [73], Bowen [16], and Montagna [83] as the Golgi elements in these cells. The sebaceous transformation occurs within the confines of the Golgi complex of vacuoles either by chemical alteration of the membrane substance or by segregation of materials produced in the interstitial cytoplasm and concentrated within the membranes. As Montagna [82] has pointed out, the mitochondria have little topographical relation to the secretory product and remain intact even in the mature cells (see Fig. 155). It appears, also, that the basophilic elements of the cytoplasm decrease only in over-all concentration as the cell becomes distended by the lipide droplets. The cytoplasm between droplets seems to be just as densely granular in mature cells as in the undifferentiated cells at the periphery of the acinus (cf. Figs. 151, 155).

THE CYTOLOGY OF SECRETION IN APOCRINE GLANDS. Although the goblet cell is usually not classified as an apocrine gland, it will be considered as such for the purposes of the present discussion for reasons which will become evident. The mature goblet cell—for example, in the intestinal epithelium—is familiar to every student of histology as a distinctive cell type not only because of its characteristic shape but also because of the staining reactions of its contents [103, 114]. In conventional sections stained with hematoxylin and eosin it appears as a slender columnar cell with a basal nucleus slightly flattened by the unstained secretory material filling the swollen apical cytoplasm above it. The secretory material stains distinctively with certain aniline dyes, such as light green or aniline blue, stains metachromatically with toluidin blue, and gives a positive reaction with the Schiff reagent after oxidation by periodic acid or lead tetracetate. These histochemical reactions indicate that the content of the goblet cell is a sulfate-containing mucoprotein. The cell evidently produces and releases its secretory product continuously [59], but may revert to a stage resembling the condition of its neighboring absorptive cells. When it has few or no secretory droplets it can still be recognized, however, by the paucity of its mitochondria and the fragmentation of its Golgi apparatus.

In electron micrographs, the resting (or exhausted) goblet cell (Fig. 156) has the usual columnar shape of its neighbors. Its lateral surfaces are thrown into numerous folds which interdigitate with the cells around it. Its apical or free surface displays a striated border consisting of microvilli of the same dimensions as those found on the absorptive epithelial cells. Its nucleus is ellipsoidal and its cytoplasmic matrix consists of a dense feltwork of interlacing delicate fibrils in which are embedded sparse, small, filamentous mitochondria, elements of the ergastoplasm, and small, widely dispersed vesicles of the agranular reticulum. A compact Golgi complex is not evident. At the onset of secretion both the ergastoplasm and the agranular reticulum proliferate. The supranuclear cytoplasm becomes crowded with broad, branching and anastomosing ergastoplasmic cisternae, oriented with their long axes parallel to the long axis of the cell. A compact Golgi complex appears in the immediately supranuclear zone, as a dense collection of more or less parallel cisternae, tubules, and vesicles of the agranular reticulum (Fig. 157). The mitochondria appear not to participate in the process by any noticeable structural change. They come to lie in the peripheral cytoplasm of the cell and are only rarely found more centrally, among the ergastoplasmic sacs or the Golgi membranes. As the goblet develops in the supranuclear cytoplasm, the Golgi complex enlarges and increases considerably in complexity. The packed agranular membranes become arranged into a flask-shaped mass standing above the nucleus (Fig. 157). The walls consist of multiple, imbricated and intercommunicating, broad, shallow cisternae forming continuous, fenestrated plates which enclose a collection of small and large vesicles. The contents of these vesicles are somewhat denser than the cytoplasmic matrix between them and resemble the droplets of mucin in the definitive secretory material.

The apex of the flasklike Golgi complex is usually capped by a thin collection of ergastoplasmic cisternae, through the meshes of which vesicles containing secretory product—the secretory droplets—escape to form the aggregation of mucin droplets that give the cell its characteristic mature shape. The ergastoplasm is displaced to the peripheral cytoplasm by the expanding Golgi complex, and around the dilated end of the cell (Figs. 158, 159) it is compressed into a thin dense wall which corresponds to the *theca* described by F. E. Schultze [117]. The droplets accumulating in the apex of the cell gradually distend it so that the folds of the surface become ironed out. Their membranes become discontinuous and neighboring droplets merge into larger masses (Figs. 158, 159). The microvilli at the surface become flattened out, and finally the apex of the cell bursts open, so that the contents of the goblet flow out into the lumen of the intestine (Fig. 159). This outpouring of the apical region of the goblet cell justifies its inclusion among the apocrine glands.

In these goblet cells, the secretory material is clearly collecting in the vesicles of the Golgi complex, as Nassanov [86] showed some thirty years ago [see also 23]. Recently Jennings and Florey [59] have given added confirmation to this conclusion. By means of autoradiographs they followed the distribution of radioactivity in goblet cells after the intravenous injection of $Na_2{}^{35}SO_4$ into mice, rats, cats, guinea pigs, and rabbits. They found that in the earliest specimens studied (one to three hours) the radioactivity was concentrated in the supranuclear region of the cell, between the goblet and the nucleus. With the passage of time (six hours) the radioactivity moved into the goblet itself, and finally (twelve to twenty-four hours) out of the epithelium. The uptake of radioactive sulfur, presumably for incorporation into mucin, by the juxtanuclear Golgi complex indicates that this organelle either synthesizes or concentrates the mucous secretory product.

In the cells of the mammary gland, a more generally accepted apocrine gland, the acinar cells shed their apical cytoplasm along with its constituents, the mitochondria, secretory droplets, and fat droplets. Large as well as small, dense secretory droplets accumulate within the agranular membranes of the Golgi complex in the supranuclear region, and as the vesicles enlarge they come to lie in the apices of the cells, where they occupy broad streamer-like processes extending into the lumen of the acinus. The bases of the processes pinch off and the resultant fragments are freed into the lumen. Single massive fat droplets are also extruded in a similar way. Perhaps the contractile action of the myoepithelial cells is important for this type of extrusion.

THE CYTOLOGY OF SECRETION IN MEROCRINE GLANDS. The cytology of merocrine secretion has been most carefully studied in the salivary glands and the pancreas. The light microscopic literature has been reviewed by M. Heidenhain [50], Schaffer [114], Bowen [16], and Saguchi [113]. The essential point in merocrine secretion is the formation of a granule [109, 110] containing the characteristic product of the cell. Nassanov [86, 87] was the first to show a clear-cut relation between these granules and the Golgi apparatus in the pancreatic acinar cell [see also 24]. He demonstrated that the secretory or zymogen granules first appeared in the supranuclear cytoplasm in the meshes of the Golgi apparatus as impregnated with osmium and that as the cell discharged its secretory granules upon pilocarpine stimulation, the Golgi network moved with them to the apical surface. New secretory granules developed in these remnants of the Golgi network, until the supranuclear zone was full once more of droplets interspersed with the extended Golgi apparatus. Nassanov regarded the Golgi network as a segregation apparatus for the products synthesized in the remainder of the cell.

The electron microscopic counterpart of Nassanov's observations is shown in Figures 160, 162, and 163, which contain small areas of the apical cytoplasm of pancreatic acinar cells from a mouse killed four hours after an injection of pilocarpine. The agranular membranes of the Golgi complex are aggregated as closely packed vesicles and vacuoles. Some of these contain irregular dense material (Figs. 162, 163), and a sequence of stages can be constructed, from the smallest clear vacuoles through intermediate vacuoles containing dense material to the definitive secretory droplets. In a study [99] of untreated and pilocarpine-stimulated pancreas in mice and rats no connection between the secretory droplets and mitochondria, ergastoplasm, or any cytoplasmic organelle other than the Golgi complex has been discovered. This conclusion is contrary to the interpretations of Weiss [128] concerning the origin of secretory granules in the pancreas of mice after prolonged fasting. As pointed out by Mathews [79] and again by Bensley [15], both the ergastoplasm and the secretory granules disappear if the animals are subjected to prolonged fasting. Soon after the first feeding, the ergastoplasm reappears in the bases of the cells and later the secretory granules return, starting as very fine prozymogen granules in the supranuclear zone. Mathews [79] suggested that the zymogen granules arose from disintegration of the basophil threads which comprise the ergastoplasm of the pancreatic cell. Although these threads were soon dismissed as artifacts of fixation resulting from swelling or dissolution of mitochondria [14, 58], they are now recognized to be the swollen elements of the granular endoplasmic reticulum or ergastoplasm (Fig. 161). In his electron microscopic study of the pancreatic acinar cell, Weiss [128] showed that the prozymogen granules first appeared after fasting as small vacuoles budding off from the reforming endoplasmic reticulum and that they matured into definitive secretory droplets as they moved toward the apices of the cells. But this study was done before a clear distinction between the granular endoplasmic reticulum of the ergastoplasm and the agranular reticulum of the Golgi complex was established. Therefore, in the light of the present study his interpretation cannot be taken as conclusive. Furthermore, the concurrent reappearance of the ergastoplasm and the zymogen granules after the first meal following upon a prolonged fast can be taken only as indication of the re-establishment of protein synthesis when the materials for synthesis are offered to the cell. From an elegant study of the topographical relations of the zymogen granules and the Golgi membranes, Sjöstrand and Hanzon [122, 123] have come to the same conclusion as the current author.[5]

The electron micrographs also provide no evidence that zymogen granules in the pancreas originate in connection with mitochondria or with any cytoplasmic organelle other than the Golgi complex (cf. light microscopic findings of Hoven [58], Lacy [61–64], and Saguchi [113]). According to Lacy [64], in agreement with Saguchi [113], the zymogen granules originate as small lipoidal bodies arising from the mitochondria. They next pass to the Golgi apparatus, and later they leave it to move toward the apical portion of the cell, where they first become vacuolated and then merge with the so-called "secretion bodies." These are sudanophilic bodies of various sizes that Lacy has described among the secretory granules in the pancreatic acinar cells of normal and temporarily starved mice. As these bodies seem to increase in number during the discharge phase of the secretory cycle, initiated by feeding or by injection of pilocarpine, Lacy [63, 64] has termed them "secretion bodies." They consist of an outer lipoidal coating and an inner substance that includes secretory granules which have coalesced with the "secretion bodies" at some time during their development. Although Lacy could not determine the method by which the "secretion bodies" were discharged from the cell, he found that the canaliculi and small ducts leading from discharging acini were full of sudanophilic material. These findings and interpretations were not corroborated by Xeros [136], who found that secretory droplets arise *in situ* as prozymogen granules and not as lipoidal bodies.

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[5] Palade (J. Biophys. Biochem. Cytology, *2*, 417–22, 1956) has reported that, in the pancreas of fasted and refed guinea pigs, dense, homogeneous, spherical bodies appear within the cavities of the granular endoplasmic reticulum (ergastoplasm). These bodies resemble the mature zymogen granules in density, but are smaller and do not possess a surface membrane. These intracisternal bodies disappear within a short time after refeeding, as the mature secretory droplets accumulate in the supranuclear portions of the cell. It is possible that in the pancreas of the guinea pig, during the initial stages following refeeding, secretory material is produced at such a rapid rate that segregation occurs within the ergastoplasm instead of within the cisternae of the Golgi complex. This phenomenon apparently does not occur in the pancreas of either the rat or the mouse (see also Farquhar, M. G., and Wellings, S. R., J. Biophys. Biochem. Cytology, *3*, 319–22, 1957).

The correlation between Lacy's "secretion bodies" (and his other lipoidal bodies) and the lipochondria of Baker is not immediately clear. Indeed, it is not entirely clear whether Lacy has found a new structure or is merely renaming the sudanophilic material which occurs in the neighborhood of secretory granules and the Golgi complex. Peculiar bodies, however, which may be the equivalents of "secretion bodies," have been found in electron micrographs of sections from the pancreas of mice that have been treated with pilocarpine [64, 99] or neutral red [129]. Lacy [64] has observed in preliminary electron microscopy of such experimental material "that during the discharge phase artificially induced by the use of pilocarpine hydrochloride the lamelliform appearance of the ergastoplasm disappears and masses of small ergastoplasmic vesicles are produced. The vesicles flow together and sometimes embrace zymogen granules to form large spheroidal inclusions which clearly correspond to the secretion bodies." In a study of the pancreas in mice that had been injected with neutral red, Weiss [129] found that the ergastoplasm undergoes focal degeneration and gives rise to lumps of homogeneous gray material. Furthermore, complex, vacuolated and granular, irregularly shaped osmiophilic inclusions appear in the cytoplasm, which apparently represent degenerated mitochondria and are equivalent to the neutral red vacuoles visible in the light microscope. All of these new formations can be seen in electron micrographs [99] of sections from the pancreas of mice that have been treated with pilocarpine (Figs. 164–166). As Lacy reports, the ergastoplasm loses its highly oriented arrangement and becomes transformed into a collection of smaller dilated vesicles (Fig. 164). Isolated bodies, consisting of ergastoplasmic vesicles and cytoplasmic matrix together with numerous nucleoprotein granules, become separated from the surrounding ergastoplasm by a clear space (Fig. 165). These bodies are not bounded by a distinct membrane but seem to represent merely a condensation of small but dilated vesicles and their associated nucleoprotein granules. In contrast to Lacy's interpretation, however, these condensed ergastoplasmic bodies do not contain secretory granules and seem to have nothing to do with the process of extrusion of secretory material.

Focal dissolution of the ergastoplasm in other regions, particularly in the base of the cell, also occurs, after pilocarpine injection (Fig. 164) exactly as described by Weiss in animals treated with neutral red. Furthermore, small and large, dense, heterogeneous bodies, usually surrounded by a single membrane, are frequently visible throughout the apical region of the cell after pilocarpine as well as after neutral red (Figs. 164, 166). As these remarkable changes, difficult to interpret in the light of present knowledge, can be found occasionally in the pancreas of normal animals, it is likely that they are not manifestations of toxic drug action but rather exaggerated physiological changes related to the exceedingly rapid rate of secretory synthesis and discharge induced by the injected chemicals.

THE CYTOLOGY OF SECRETION IN ENDOCRINE GLANDS. The secretory process in the endocrine glands is similar to that in the other merocrine glands. Granules, presumably containing the specific hormones, appear in the cytoplasm and are extruded in an unknown fashion to enter the blood stream instead of ducts. Study of the process, however, is rendered difficult because the cells in endocrine glands do not undergo cyclic alterations of granule production and extrusion. The only available method of tracing the origin of granules is to note topographical relations between granules and other cytoplasmic organelles in cells of animals that are subjected to different physiological states. This has been done most thoroughly in the adenohypophysis, thyroid, and adrenal cortex.

In the adenohypophysis secretory granules are dispersed throughout the cytoplasm of the chromophile cells. In the acidophiles the granules are usually about 350 mμ in diameter and in the basophiles they are 140–200 mμ in diameter [111]. In sections examined in the electron microscope (Figs. 167, 168) [35, 36, 45, 118, 99], the Golgi complex commonly appears as a prominent, more or less circular, juxtanuclear area containing arrays of closely packed, oval or elongate profiles that represent membrane-limited cisternae, tubules, and vesicles. These are often, but not always, arranged around the periphery of a cytoplasmic region containing numerous small vesicles and secretory granules of various sizes (Figs. 167, 168). Sometimes the broad cisternae extend from the periphery across this region and are dilated to form large vacuoles. Occasional mitochondria and small bits of ergastoplasm also lie in this region along with infrequent, rounded, membrane-bound bodies of heterogeneous density (Fig. 167). In the chromophobe cells, which contain few or no granules, the Golgi complex is small and inconspicuous [111]. The close topographical association of the Golgi complex and the secretory granules in the chromophiles again suggests that the latter originate in the vesicles of the Golgi complex (Figs. 167, 168). Following castration or thyroidectomy, the Golgi complex of the basophiles undergoes distinct changes [35, 36, 118]. It enlarges considerably and the number of small vesicles within the Golgi area increases. As the number of secretory granules decreases with time after operation, they appear to be congregated in the Golgi zone. Farquhar and Rinehart [35] suggest that these changes indicate participation of the Golgi complex in both synthesis and release of the secretory material.

The origin of secretory granules in the thyroid gland has been considered in only two electron microscopic studies [34, 130]. Although other authors [17, 80] have investigated the fine structure of the thyroid gland, they did not identify the Golgi apparatus or clearly delineate secretory droplets. Dempsey and Peterson [34] described dilated ergastoplasmic sacs containing a homogeneous, dense substance which they identified as the secretory product of the thyroid, i.e. the colloid. They believed that the colloid accumulated within the ergastoplasmic sacs. As the contents of the sacs became denser, the sacs rounded up into spherical droplets and the granules of ribonucleoprotein [93] disappeared from their outer surfaces, so that eventually a fully formed secretory droplet enclosed in a smooth agranular membrane resulted. They concluded that the granular endoplasmic reticulum, or the ergastoplasm, is the source of the colloid droplets.

A more recent and thorough study of secretion in the thyroid follicular cell came to a quite different conclusion [130]. This investigation took into consideration the complex physiology of the thyroid cell, which combines both exocrine and endocrine phases. The secretory droplets, in this gland the so-called *colloid droplets,* are uniformly dense, membrane-bound, rounded bodies of various sizes that lie in the supranuclear cytoplasm (Figs. 169, 170). The smallest of these droplets are always associated with the vesicles of the Golgi complex, which in the thyroid follicular cell is usually a very dense aggregate of small spherical vesicles and a few elongated shallow cisternae occasionally exhibiting localized dilatations (Figs. 169, 170). The small Golgi vesicles contain material of varied electron density. In some instances it is so dense that the vesicles may appropriately be described as granules. In the first few hours after the follicular cells have been stimulated by exogenous thyrotrophic hormone, the Golgi complex hypertrophies and the number of colloid droplets in the cells increases. A complete series of droplets intermediate in size and density between the mature or definitive colloid droplets and the small spherical vesicles of the Golgi complex can commonly be found (Fig. 170). Later, tabs of cytoplasm containing secretory droplets can be seen extending into the extracellular colloid within the lumen of the follicle. Apparently the colloid droplets are extruded through these streamers into the lumen. The microvilli covering the apical surface of the cells decrease in size and number, and the cell seems to be in an excretory phase. With prolonged stimulation the vesicles of the Golgi complex proliferate enormously and intrude into all parts of the perinuclear and apical cytoplasm. Numerous intermediate forms between the Golgi vesicles and the mature secretory droplets are seen.)

When the endocrine phase of the thyroid secretory process is exaggerated by chronic administration of propylthiouracil in the diet of the animals or by subcutaneous injection, the Golgi complex becomes hyperplastic but the number of mature secretory droplets is small. The follicular lumen becomes smaller as the colloid is withdrawn into the circulation. Correlated with the reduction in the amount of follicular colloid are an increase in the height of the follicular cells, an increase in height and complexity of the microvilli at their apical surfaces, an increase in the number of minute subapical vacuoles (Fig. 171), and tremendous dilatation of the ergastoplasmic sacs, which become the most prominent element of the cytoplasm (Fig. 172). The sacs are filled with a homogeneous material slightly denser than the cytoplasmic matrix and resembling the intrafollicular colloid. As transitions between the subapical vacuoles and the dilated ergastoplasmic sacs can be found, it appears that the colloid is being absorbed from the follicular lumen by a process akin to pinocytosis and then is transported to the ergastoplasm through which it passes to the base of the cell for discharge into the circulation. These dilated ergastoplasmic sacs, which also appear in smaller numbers in normal thyroid cells (Fig. 169), were incorrectly identified by Dempsey and Peterson [34] as the colloid droplets, whereas the definitive, mature colloid droplets were evidently not recognized.

In the normal thyroid cell these two processes of colloid formation and resorption must proceed more or less simultaneously. The experimental stimulation of the cells by endogenous (effect of feeding propylthiouracil) or exogenous (injections of thyrotrophic hormone) pituitary hormone permits a physiological separation of the two processes that aids in the attribution of function to the various cytoplasmic organelles. It is clear from this study [130] that the colloid droplets arise from the collection of colloid within the small Golgi vesicles (Fig. 170), which enlarge and move toward the apex of the cell from which they are discharged into the lumen. The ergastoplasmic sacs apparently serve as a pathway for resorbed colloid transported to the base of the cell.

The adrenal gland has not been thoroughly studied by electron microscopy. Only one study has appeared on the adrenal cortex [70] and one on the adrenal medulla [71]. Secretory droplets were readily distinguished in the cells of the medulla as dense homogeneous bodies surrounded by a smooth membrane, but the author could not decide whether they derived from the ergastoplasm or the Golgi vesicles, although he inclined toward an ergastoplasmic origin. In the cortical cells the situation was much more complex. Dense lipide droplets of various sizes clearly appear within the mitochondria, and it was suggested that the mitochondria are the sites of elaboration or accumulation of lipides. Since steroids are lipide-soluble, the evidence suggests that the mitochondria are implicated in the formation of the cortical steroids. An additional saccular structure dispersed like the cells of a honeycomb through the cytoplasm was identified tentatively as the Golgi complex. If the interpretations of this study are correct, the cells of the adrenal cortex would be the only known exception to the generalization that secretory materials are produced in association with the Golgi complex. Obviously much more work must be done before the cytological development of secretory material in this gland is clarified.

3. Summary Hypothesis

The foregoing survey of glandular secretion indicates that the Golgi complex is the cytoplasmic organelle most consistently associated with emergent secretory granules. Even more convincingly than the light microscopic observations of the past fifty years, the recent electron microscopic studies document the close relationship between secretory granules and the membrane-bound vesicles and cisternae that constitute the Golgi complex. However, the precise role of the Golgi complex remains obscure. Does the secretory granule arise within the Golgi vesicles as a result of their specific synthetic properties, or is the secretory substance produced elsewhere in the cell and merely segregated within the vesicles? This important question is somewhat closer to an answer now than it was in 1929 when Bowen's review [16] was published. So far as protein secretory materials are concerned, it seems probable that they are produced elsewhere. Recent histochemical and cytochemical studies indicate quite conclusively that protein synthesis is clearly a function of the basophilic components of the cytoplasm, in other words, of the ergastoplasm. Morphologically, this organelle is the system of intracellular cisternae and vesicles of endoplasmic reticulum that are encrusted with fine particles or granules of ribonucleoprotein [93, 94, 97, 105]. It is specifically the latter component that is involved in protein synthesis, for isotopic studies have demonstrated that amino acids are more rapidly incorporated into these granules than into any other cellular component [72]. A recent preliminary study [120] of the pancreas reveals that intravenously administered leucine is most rapidly incorporated into the protein of the microsome fraction (derived by differential centrifugation from ergastoplasm [98]), next most rapidly into the mitochondrial fraction, and more slowly into the secretory granules. As the specific activity of the microsome fraction declines, the specific activity of the secretory granules continues to rise, whereas that of the mitochondria remains relatively static. Although the experiment gives no direct information about exchange of molecules between these organelles, it is tempting to speculate that the declining microsome activity together with the rising secretory granule activity indicates transference of the protein synthesized in the microsomes to the newly formed secretory granules.

Although isolated Golgi bodies display some enzymatic activity, such as phosphatase, it is unlikely that they are capable of protein synthesis. It seems evident that they must be considered primarily as segregating systems capable of collecting and sequestering substances produced elsewhere in the cell and brought to them either by the continuous streaming of the cytoplasm or by diffusion across the matrix. Probably, as Bowen [16] indicated, the whole cell, including the nucleus, mitochondria, and the ergastoplasm, is involved directly and indirectly in the synthesis of the ultimate secretory product, which is selectively collected in the vesicles of the Golgi complex to form the definitive secretory droplet. Biochemical evidence suggestive of cooperative participation in certain enzymatic reactions is already available [115].

Such considerations permit the development of a comprehensive hypothesis for the topographic segregation of cytoplasmic functions. The endoplasmic reticulum, which is in dynamic continuity with the plasmalemma and the nuclear membrane [94], serves as a transportation system for conveying raw materials from the cytoplasmic environment to the enzymatic machinery which resides in the ergastoplasm and the mitochondria. The products of these systems are released into the cavities of the endoplasmic reticulum or into the matrix of the cytoplasm, whence specific substances are sequestered in the Golgi complex as secretory granules to be extruded from the cell as the ultimate secretory product. The documentation of this hypothesis requires much further investigation invoking the results of both morphological and cytochemical experiments.

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12. *The Cytology of Striped Muscle*[1]

BY H. STANLEY BENNETT

IN THIS DAY the term "analytical cytology" connotes a branch of biology replete with complicated optical and electronic instruments, measurements, advanced mathematical treatments, and chemical fractionation and analysis, with concepts cutting across traditional boundaries of scientific fields. The spectacular advances in our knowledge of cytology now taking place evidence the fruitfulness of modern approaches. Yet there were persons who utilized the same interdisciplinary approaches a century ago and who observed with such discernment and understanding that their writings can convey inspiration and conceptual guidance today. Such penetrating and prescient observers were Bowman, Kühne, Engelmann, Ranvier, Kölliker, and Cajal. In reviewing the cytology of striped muscle one can see that many of today's concepts were formulated by the nineteenth-century giants of analytical cytology.

1. On the Nature of Muscular Contraction

Striped muscle is a specialized tissue which has developed to a high degree a property present in many cells—contractility. This can be regarded as the ability to produce a rapid and reversible change in the elasticity of its structure. Such an increase in the elastic constants need not result in appreciable shortening. If the ends of the structure are anchored firmly enough, a contraction is isometric. In this case the force exerted by the structure at a given fixed elongation is increased. We can use Hooke's law to illustrate this point, bearing in mind that it does not describe the elastic behavior of muscle very well (see [25]). Hooke's law can be written

$$\triangle L = kF$$

where $\triangle L$ is the elongation, F is the deforming force, and k is a constant. A muscle contraction can be regarded as resembling an increase in *k*. If the length is not permitted to change on contracting, *F* will increase.

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Muscular contraction is believed to be accomplished by the direct conversion of chemical energy into mechanical energy without the intermediate production of heat. If the muscle is loaded and if shortening occurs, kinetic energy is produced, and the muscle does work—as a physicist speaks of work. If no shortening occurs, the chemical energy is converted into mechanical potential energy.

For a century or so it has been realized that this contractility must depend on unusual properties in the molecules comprising contractile units in muscle. Kühne [84] in 1859 introduced the word "myosin" to apply to a proteinaceous material which he expressed from muscle in the course of his effort to explore the chemical basis for contraction. As knowledge of protein chemistry has been sharpened, this term has been redefined from time to time [33, 94, 95, 130]. "Myosin" remains, as always, a term applied to a protein found in abundance in muscle and thought to have an important role in contraction. However, other proteins—actin, for example, first recognized by Straub [130, 131], and the tropomyosin of Bailey [8]—are also suspected of contributing significantly to the contractile process.

Excellent reviews of muscle protein chemistry and of concepts regarding the molecular basis for contraction have been published by Kühne [85], Weber [143], Fenn [42], Weber and Portzehl [144], and Bailey [9]. Polonovski [107] has presided at a symposium, the proceedings of which provide a valuable summary of contemporary viewpoints relating to muscle physiology and biochemistry. These reviews show that great progress has been made in fractionating and characterizing the proteins of muscle.

Yet it is clear that we have no satisfactory understanding of the chemical basis of the contractile process, although some bold and fruitful speculations have been injected into the literature from time to time. Perhaps the most important is that of Meyer [91], who suggested that reversible introduction of charged groups on a linear fibrous protein molecule would change the elastic constants of the fiber reversibly and provide a chemical basis for a contractile system. Kuhn [82] and Kuhn, Hargitay, Katchalsky, and Eisenberg [83] showed that a model contractile fiber system could indeed be synthesized, made to shorten reversibly, and do work in accordance with principles very similar to those suggested by Meyer. Fischer [44], Buchthal [24], and others have suggested variants of the Meyer hypothesis as bases for muscular action. Yet there is no real evidence that muscular contraction is based on the principles enunciated by Meyer, and some have thought his suggestion an unlikely basis for contractility, since the force-extension curves of muscle do not follow the inverse square law, which should apply if coulombic forces operated simply in a linear array parallel to the long axis of the fiber [25]. However, as Buchthal and Kaiser have pointed out, the structure of muscle is complicated, the contractile structural molecules constitute only one of several components contributing to the elastic characteristics of the whole fiber, and a number of geometrical arrangements of the contractile molecules are possible in addition to a strictly linear one. Thus there does not seem to be reason to abandon altogether Meyer's hypothesis as a possible molecular mechanism having a role in muscle contraction.

2. *The Place of Striped Muscle among Contractile Tissues*

Most biochemists and physiologists interested in basic problems of contractility have focused their attention on striated muscle. Huxley and Hanson [73] and Hanson and Huxley [54] have proposed a model for contraction of muscle which depends on the organization of muscle into cross-banded units (Text Fig. B). Yet it must be remembered that striped muscle is only one of a number of types of contractile tissue or structures, and any general theory of contraction will be more useful if it can be applied to all of these, rather than to some restricted type of muscle only. Thus we must search for a common molecular basis for the activity of all contractile tissue and then try to discern the way in which this basic pattern may be modified or adapted in the special cases of striated and smooth muscle, sperm tails, cilia, and other contractile structures.

3. *Distribution of Striped Muscle in the Animal Kingdom*

Although striated muscle appears to be a highly specialized type of contractile tissue, it is present in relatively primitive animals and can be regarded as a fairly ancient biological invention. It can be found in coelenterates—in the circumumbrellar muscle of certain jelly fish, for example. Heidenhain [62] reports that striated muscle occurs sporadically in coelenterates, echinoderms, bryozoa, molluscs, salps, chaetognaths, and appendicularia. Hartog [58] states that striped muscle exists in many free-swimming rotifers. Schmidt [123, 124] has described in chaetognaths of the genus *Sagitta* a peculiar type of striated muscle showing negative birefringence in the I band. Haswell [60, 61], Okada [97], and Schmidt [122] have described radial striped muscle surrounding the proventriculus of certain polychaete worms of the family *Syllidae,* in which the sarcomeres achieve unusual lengths of 20 to 30 or even 100 microns. In most invertebrate phyla, striated muscle appears to be distributed somewhat capriciously, occurring here and there in places where smooth muscle is found in closely related forms. In the scallop, *Pecten,* striped muscle serves the function of activating the shells in swimming, whereas a smooth muscle is used to hold the two shells together in sustained closure. In arthropods all muscle is striated, and striped fibers serve as visceral motive agents for the digestive tract and Malpighian tubules, as well as for skeletal motion. Striped muscle is generally present in all vertebrates.

4. General Architecture of Striated Muscle

The striped muscle of vertebrates and of arthropods, particularly of insects, has been studied extensively, and a remarkable uniformity of basic architecture has been substantiated. There remain, however, many invertebrate striated muscles which have been studied so little that the number and distribution of muscles whose cross striations differ from the usual pattern is not known. Those in *Sagitta* described by Schmidt [123, 124] comprise examples, however.

The basic architecture of striated muscle was well described by Bowman [20], whose remarkably accurate observations and discerning interpretations of insect and vertebrate muscle deserve the careful and admiring attention of every student of muscle structure. Bowman observed that each vertebrate and insect muscle fiber was encased in "a tubular membranaceous sheath of the most exquisite delicacy, investing every fasciculus from end to end, and isolating its fibrillae from all surrounding structures." This membranous sheath Bowman named the *sarcolemma,* and his term has enjoyed wide usage in the literature as applied to a membrane surrounding muscle fibers. The sarcolemma was recognized by Schwann [125] and Bowman in both vertebrate and arthropod muscles. Closely applied to the outer surface of the sarcolemmal membrane of vertebrate striped muscle is a loose plexus of delicate collagenous or reticular fibers in which lie capillaries. In arthropod muscle no connective tissue fibers invest the sarcolemma, but tracheal end cells are often closely applied to its outer surface and may provide tracheolar connections to the muscle fiber itself. The sarcolemma encloses the cytoplasmic and nuclear portions of the striped muscle. Usually several to many nuclei are present within one sarcolemmal membrane, but in exceptional cases only one nucleus is found.

Bowman [20] described and measured the dimensions of single muscle fibers or "primitive fasiculi" ensheathed by the sarcolemma. In length they range from a few microns in short muscles of insects and other invertebrates to several centimeters in the long muscles of vertebrates. In diameter they range from 10 to 20 microns at the small limit to 100 or 200 microns, or even more. In cross section the fibrils are irregularly polygonal, tending toward the hexagonal.

It is customary and convenient to divide the contents of the sarcolemma into nuclei, sarcoplasm, and myofibrils. Each of these has definite subcomponents, and the myofibrils and sarcoplasm are characteristically organized in a transverse periodicity repeating longitudinally along the fiber. In most cases the sarcolemma encloses a single, prismatic, cylindrical or fusiform fiber, usually with a length many times its diameter. In vertebrate striated muscle derived from the branchial arches, as in pharynx, palate, and tongue, branched striped muscle fibers are encountered. In vertebrate cardiac muscle a branched anastamosing network of fibers is found [62]. Although this network is usually stated to constitute a single syncytium, Werner [146] and von Palczewska [100] have advanced the concept that the intercalated discs of heart muscle may in fact divide it into numerous cellular domains, each with a few nuclei. Recently Sjöstrand and Andersson [129] and Poche and Lindner [106] have revived interest in this view, presenting evidence obtained with the electron microscope that the sarcolemma or cell membrane does indeed cut across the fiber at the intercalated disc. Thus each disc is held to contain two membranes. The heart muscle network of the crayfish, *Astacus,* has been shown by Holmgren [68] to be divided by intercalated discs into cellular domains, each containing a few nuclei, much as postulated by Sjöstrand and Andersson [129] for vertebrate cardiac muscle. Holmgren's figures 61 to 64 demonstrate this concept for arthropod heart muscle.

In many insect and other air-breathing arthropod muscles the fibers are penetrated by tracheoles which ramify extensively within the sarcoplasm and carry oxygen directly to the interior of the muscle fiber. These intracellular tracheoles have been known for a century and are extensively described in the light-microscope literature [6, 29, 79, 81, 92].

5. The Sarcolemma

A membranous sheath surrounding the muscle fiber can be seen with the electron or light microscope. This sheath separates its contents from the connective tissue fibers without. Schwann, in 1839 [125], described this sheath in the following words, quoted from Smith's translation [126, p. 138]:

> . . . the muscular fasciculi are, as it seems, enclosed by a structureless membrane. The fasciculi have been long described as invested by a sheath, but that investment has been considered to be composed of cellular tissue,[2] and to correspond in the primitive fasciculi to the cellular tissue, by which the larger fasciculi are separated from each other. This membrane seems, however, to have quite a different signification, and to be the cell-membrane of the secondary muscle-cell. It is structureless, very transparent, and appears as a very narrow and sharply-defined border around each primitive fasciculus. I well know how readily such an appearance is produced by a mere optical deception, and that one can never be positive with respect to it unless it be observed that the margin in question does not accurately follow every bend of the fasciculus. It is, therefore, difficult to be convinced of this in

[2] The term "cellular tissue" was used in the days of Schwann and Bowman to refer to connective tissue. The term "cellular membranes" was similarly applied to membranous forms of connective tissue. Thus Henry Smith [126], in translating Schwann's treatise [125] in 1847, used "cellular tissue" as synonymous with "areolar tissue" (p. 110). Again, Da Costa, in appending a note on p. 94 to Busk and Huxley's 1854 translation of Kölliker's *Manual of Microscopical Anatomy*, includes the comment, "loose or areolated connective tissue is generally described as areolar tissue, or under the faulty name of cellular tissue." See also Dunglison's Medical Dictionary [36], 1860 edition, p. 183.

> mammalia; but in all those larvae of insects which present the broad transverse striae of the fasciculi, discovered by Müller, the membrane, when the continuity of the proper muscular substance of a primitive fasciculus has been broken at a certain point, may be distinctly observed passing over uninterruptedly from the one portion to the other. Pl. IV. fig. 4, represents such a fasciculus; the membrane encompasses it so loosely (this larva had been preserved in spirits of wine) that a portion of the muscular substance could even change its position within the cavity. The membrane, where entirely isolated from the other part of the preparation, shows itself to be quite structureless, and, indeed, the sharply-defined external contour renders it very improbable that it should be composed of areolar tissue. I, therefore, consider it extremely probable that it represents the cell-membrane of the secondary muscle-cell. It thus not only serves to isolate the fasciculi, but forms an essential constituent part of them.

Bowman [20] perceived this membrane independently (see Fig. 175), but recognized Schwann's prior discovery. He confirmed Schwann's description on vertebrate and insect muscle, studied the permeability properties of the membrane, and named it the sarcolemma. Bowman described and characterized the sarcolemma so well that his concept has dominated the subsequent literature and can be taken over almost without modification for a satisfactory interpretation of electron micrographs of muscle. In agreement with Schwann, Bowman regarded his sarcolemma as being distinct from "that general sheath of filamentous tissue which invests the whole muscle and its lacerti." [3] He states further (p. 475), ". . . the true structure about to be described has no analogy whatever with the cellular membrane of muscle," and goes on to say (p. 478), "Its external surface seems to be quite smooth and free, being in contact with the capillary and cellular network of the organ, which embraces each fasciculus in more or less profusion, according to the animal and the particular muscle." He explored the osmotic properties of the sarcolemma, showing that it can be lifted from the contained fibrillar material if the muscle fiber is placed in water, and that the water so imbibed and enclosed within the sarcolemma can be removed if the fiber is subsequently immersed in syrup. In this respect its behavior resembles the plasma membrane of erythrocytes.

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[3] The word "lacerti," from the Latin *lacertus*, meaning brawn or muscle, was used in the midnineteenth century to apply to fasciculi or small bundles of muscle fibers [36].

The sarcolemma was recognized and described repeatedly by many nineteenth-century authors, who appeared to understand the membranous nature of the structure with which they were dealing [28, 79, 80, 81, 90, 115, 116, 117]. Since the turn of the century, however, confusion has entered into the terminology, and some authors have attempted to use the term "sarcolemma" for connective tissue components, which Schwann and Bowman explicitly excluded in their definitions. Thus Gutmann and Young argue as follows [49, p. 16]:

> Presumably there is a cell membrane at the surface of the sarcoplasm which is responsible for maintaining the difference in concentration between the outside and the inside of the fibre, separating a fluid rich in Na^+ and Cl^- outside from the K^+ space within. This membrane must be located at the outer edge of the thin band of sarcoplasm mentioned above, but there is no reason to suppose that it is a thick, visible membrane. Under favorable circumstances the edge of the sarcoplasm can be clearly seen, but presents no special membrane. The layer which is usually called sarcolemma is quite a thick membrane that lies more peripherally, and probably does not strictly belong to the muscle fiber but to the endomysium. There is no reason to suppose that this thicker layer has special permeability properties. We may therefore reasonably suppose that the muscle fiber is surrounded by two membranes: (1) a thin but physiologically important limiting membrane of the sarcoplasm, which may be called the muscle membrane or sarcoplasmatic membrane, and (2) a thicker, visible layer, of the nature of connective tissue, the sarcolemma.

Péterfi [105] is cited in support of this argument.

Apparently Gutmann and Young failed to achieve what Bowman accomplished a century before them, which was to demonstrate the connective tissue structure, the muscle fiber membrane, and the sarcoplasm and myofibrils as separate entities. They appear unaware that Bowman had demonstrated semipermeable properties of the membrane he called the sarcolemma, and that a thin membrane with a thickness below the limits of resolution of a light microscope can nevertheless be rendered visible by virtue of its diffraction effects, if separated from neighboring structures by a resolvable distance. Thus they were led into the unfortunate practice of assigning a new term, the "sarcoplasmatic membrane," to the structure which Bowman called the sarcolemma and which they did not demonstrate with their "ordinary" methods, and of using Bowman's term, sarcolemma, for connective tissue structures which Bowman recognized as quite distinct from and outside of the sarcolemma as he saw it. Barer [10], in his excellent review, recognizes this confusion and uses the term sarcolemma correctly, in accordance with Bowman's concepts and the customary usage in the classical light-microscope literature on muscle.

Recent electron micrographs of muscle fibers can be regarded as confirming Schwann's and Bowman's brilliant observations and interpretations. Figure 173 shows the sarcolemma as it appears on a flight muscle fiber from the giant European diving beetle, *Hydrophilus piceus*.[4] The sarcolemma appears as a membrane about 1000 A thick. On closer scrutiny it displays two dense layers with a lighter lamina between. We can call the two dense layers the inner and outer sarcolemmal laminae. Each is about 200 A thick in this form. The less dense sarcolemmal interlamina is about 600 A thick in *Hydrophilus* flight muscle. A similar sarcolemmal lamination has been described in grasshopper leg muscle by Bennett [14], and tracheoles have been observed to ramify between the inner and the outer laminae. In insects there are no connective tissue fibers closely applied to the outside of the sarcolemma (see Fig. 173).

The sarcolemma of vertebrate muscle fibers is more delicate, but appears to be similar in basic architecture. Bennett [15] has described the sarcolemma of mouse gracilis muscle as being about 100 A thick overall, also with a dense inner and outer sarcolemmal laminae, and with a less dense interlamina about 40 A thick. In vertebrate muscle Reed and Rudall [111], Bennett and Porter [13], and Bennett [15] have shown with the electron microscope that the collagenous or reticular fibers corresponding to Bowman's "cellular membranes," "cellular network," or "filamentous tissue" form a loose investment outside the sarcolemma. These filaments have been termed "sarcolemmal collagen," to designate them as connective tissue fibers associated with but outside the sarcolemma, just as Bowman described them.

Jones and Barer [74] and Reed and Rudall [111] have studied the sarcolemma of vertebrate muscle on surface view with the electron microscope and have shown it to be stigmatized with numerous rounded, raised areas or with spots of density greater than that of the surrounding membrane. These spots are 400–1,000 A in diameter and are spaced irregularly about 5,000 A apart. Very similar raised stigmata have been seen on the lamellations of the myelin sheath of nerves by Fernández-Morán [43].

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[4] The author wishes to thank Professor J. T. Randall, F.R.S., and Dr. Jean Hanson of the Biophysics Research Unit, Wheatstone Physics Laboratory, King's College, University of London, for hospitality during the summer of 1954, for the use of laboratory facilities, and for providing the specimens of *Hydrophilus* used in this study.

The inner surface of the sarcolemma was shown by Melland [90] in 1885 to be adorned with markings corresponding in position to the level of the Z bands. These markings were studied extensively with the light microscope by Chlopkow [31], who regarded the structures as thin fibers running mainly in a cross direction and corresponding to the Z "discs." One of Chlopkow's illustrations is reproduced in Figure 174. Later Draper and Hodge [35] observed these markings with the electron microscope, describing them much as did Chlopkow. Bennett and Porter [13] and Bennett [15] noted them in sectioned muscle and recognized them as attachments to the inner surface of the sarcolemma of strands of sarcoplasmic reticulum—a component of the muscle fiber extensively described by Retzius [114, 115], Cajal [28], Veratti [137], Rollet [116, 117], and others, but largely ignored since Heidenhain's classic monograph of 1911 [62].

One may attempt a comparison of the fine structure of the sarcolemma with the "plasma" or "cell" membrane, now seen frequently in electron micrographs of other types of cells. The plasma membrane as it is usually resolved in columnar epithelial cells is well described by Yamada [147]. The ordinary plasma membrane appears in his sections as a single structure and is more delicate than the sarcolemma of muscle cells. Raised or dense spots and regular internal markings have not been detected in an ordinary cell membrane, although the "terminal bars" do represent a type of specialization. Very recently Brandes, Zetterqvist, and Sheldon [21] have reported that the cell membrane overlying the microvilli of the intestinal cells of the mouse is a double structure consisting of two dense sheets each about 40 A thick, between which is a less dense zone 25 A thick. If these observations are confirmed, a close parallel between sarcolemma and cell membrane of intestinal cells may emerge.

The sarcolemma was shown by Bowman to have semipermeable characteristics and to behave osmotically like the cell membrane of erythrocytes. One cannot say on the basis of present evidence whether the sarcolemma as a whole serves as the cell membrane spoken of by Schwann [125] and by Gutmann and Young [49], or if one of the laminae comprising it serves this purpose. Ling and Gerard [86] have shown that there is a membrane surrounding the muscle fiber with remarkable electrical properties, maintaining a potential difference of 70 to 100 mv. between the inside and the outside of the fiber. The sarcolemma of Bowman, with its special properties of permeability, and with the fine structure described here, is the only structural member to which one can reasonably attribute these electrical properties. If this is valid, it follows that a sarcolemma 100 A thick, as in vertebrate muscle fibers, and maintaining a potential difference of 100 mv., would have to be capable of withstanding a voltage gradient of 100,000 volts per centimeter. If one of the laminae of the sarcolemma is in itself responsible for this separation of charge, the potential gradient would be correspondingly greater. Bowman demonstrated the sarcolemma to possess remarkable mechanical and elastic properties. He showed it to be tough and strong, to have no preferential tearing axes, to be separated easily from the fibrils, and to be capable of a considerable degree of stretch without breaking—in this property exceeding the capabilities of the myofibrils themselves.

As Schwann and Bowman demonstrated, the sarcolemma can be separated readily from the contained myofibrils, which lack any firm attachments to it (see Fig. 175). It would appear to offer some promise of reward to a worker who might wish to separate a comparatively large sample of sarcolemmal fragments from muscle by procedures of differential centrifugation, which have been useful in separating mitochondria, nuclei, and other cellular fractions (see Hogeboom, Schneider, and Striebich [67]). A study of the chemical properties of sarcolemma might offer fruitful correlation with its remarkable electrical and mechanical properties.

6. The Contractile Substance

Bowman [20] showed that the fibers contained bundles of fibrillae, or myofibrils as we now call them. Kölliker [80, 81] showed that between the fibrils was a proteinaceous material or sarcoplasm, and that in many muscles the myofibrils are gathered into loosely defined anastomosing and branching groups which he called "Muskelsäulchen," or muscle columns. The columns are separated by pads of sarcoplasm thicker than those which intervene between the individual myofibrils within a column. Bennett and Porter [13] found Kölliker's concept of muscle columns useful in describing the arrangement of myofibrils in the breast muscle of the domestic fowl, as studied with the electron microscope. Kölliker [80] showed that in cross sections the muscle columns, when present, are recognizable as the fields of Cohnheim [32].

But there is great variation in the geometrical arrangement of myofibrils within a fiber. Myofibrils may be distributed fairly evenly throughout the cross section of the fiber, with more or less peripheral nuclei, as in most vertebrate voluntary muscle. Or a central core of nuclei and sarcoplasm bare of myofibrils may characterize the muscle, as in vertebrate cardiac muscle and in many insect muscles. In some cases the myofibrils are confined to one side of the muscle fiber, the rest being occupied by sarcoplasm and nuclei, as in the fiber shown in Figure 189 from von Boga's important study [19]. Retzius [114], Rollet [116], and others show cross sections of flight muscles of insects in which the muscle columns are arranged in strikingly regular concentric rows of ribbons, with the wide axis of the ribbons running radially. Still other variants have been described.

Yet in all striped muscles there appear to be definite contractile units, the myofibrils, which range from 0.5 to 5 or 10 microns in their irregularly polygonal cross sections, and which, as Bowman showed, carry the cross-banding markings of the whole fiber. Some electron micrographs of cross sections of myofibrils are shown in Figures 173 and 184. Additional ones are shown by Bennett and Porter [13], Morgan, Rosza, Szent-Györgyi and Wyckoff [93], Hodge [65], and Huxley [71].

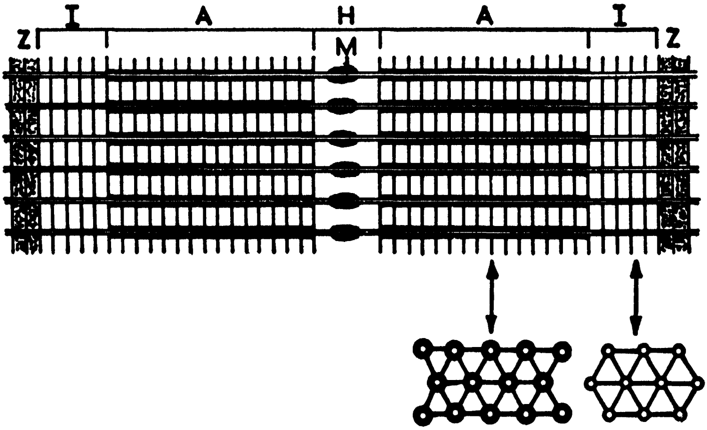
The myofibrils clearly contain the molecular contractile mechanism of muscle. Isolated myofibrils have been made to contract by addition of ATP to the medium [5, 52, 103, 118]. Harman and Montague [57] have shown elegant phase motion pictures of contracting and relaxing myofibrils of vertebrate muscle, sustained alive in a suitable nutrient medium. Hasselbach [59], Bailey [9], and Hanson and Huxley [53] have cited evidence that actin and myosin are located in the myofibril. Perry [104] found tropomyosin, myosin, actin, adenine nucleotides [102], and residual insoluble protein in the myofibril.

Striped muscle myofibrils, wherever found, with few exceptions resemble each other closely with respect to banding pattern and changes on cross banding occurring with contraction. The length of this microscopically visible repeating period responsible for the banding does vary considerably, as sarcomeres over 100 μ long are found in some syllids [60], whereas a corresponding value of about 2 to 3 microns is evident in most relaxed vertebrate sarcomeres. The relaxed sarcomere periodicity in insects and other arthropods varies from 2 to 10 μ.

But whatever the periodicity may be, the pattern sequence of bands comprising the repeating sarcomere is similar in most forms. Rare exceptions are known, as in *Sagitta* [123]. Typical relaxed sarcomeres are pictured in Figures 176, 180, 182, 187–9, 193. The sarcomere is traditionally defined as being bounded at each end by a peak in density called the Z band. Progressing along the fibril from the Z, one encounters a trough called the I, a rise in density towards the Q or A band, often with a shoulder along this rise—the shoulder known as the N band. The central 80 to 60 per cent of the relaxed sarcomere is generally occupied by a peak in density, the Q or A band, which often shows a shallow trough in its center, the H band. This in turn may show a peak at the middle of the sarcomere, the M band. The M band is the band which is seen with the least frequency in relaxed fibers.

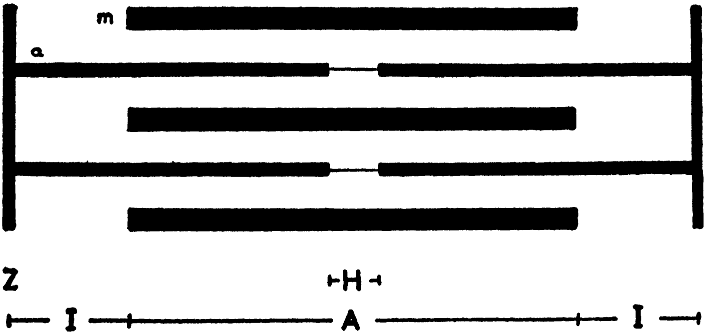
Schmidt [119, 120, 122] and Bennett and Porter [13] have demonstrated that the transitions between the cross bands are gradual and that the bands are usually rounded or peaked maxima and minima of density or of birefringence, rather than broad flat areas of a given density. The latter view is engendered by the contrast between the cross bands of the myofibril as seen in a light microscope and has created a "square wave" hypothesis, which holds that there is a sharp and definite border demarcating one band from its neighbor, with a very sharp transition, so that a scanning of density along the fibril would yield a series of levels with nearly vertical rises or descents between. This view is used by Engelmann [38], one of whose figures is reproduced here as Figure 182. A similar square wave hypothesis is implicit in Text Figures A and B, illustrating the concepts of Hodge [65] and Hanson and Huxley [54]. Tracings by Bennett and Porter [13] of the relative densities of electron micrographs of myofibrils, and studies by Schmidt [122] of the gradual transition between the A and I bands in the 55 micron-long sarcomere in the proventricular muscle of *Eusyllis* show that the appearance of gradual transition between bands cannot be dismissed on the basis of the resolving limitations of the light microscope. The gradual sloping transitions between bands make it difficult to assign sharp limits to the bands or to determine precise values for band lengths along the fiber.

In general, the sarcomere periodicity repeats itself many times along the course of the myofibril. There are, however, very short muscles in insects which consist of only a few sarcomeres. Haswell [60] portrays striated muscle fibers of *Syllis nigropuncta* as appearing to consist of two half-sarcomeres, each about 40 μ long, with a Z band running across the middle of the fibrils. These fibers seem to attach at each end at a point corresponding to the M bands. Fibers from *Polynoe,* about 100 μ long, are shown as if the whole fibril were half a sarcomere, isotropic in one half and anisotropic in the other, and were inserted at positions corresponding to the Z at one end and at the M at the other.



TEXT FIGURE A. MODEL OF THE SARCOMERE after Hodge [65]. Myofilaments in A, I, and Z are represented as bound in hexagonal array about 450 A apart by filamentous cross bridges completing the hexagons, and spaced at intervals of 250 A axially along the fiber. The myofilaments run continuously through all cross bands, but are thicker in A and M bands than in I or H. The continuous slender filament running through all bands is believed to be actin. The heavy encasement of this filament in A band is thought to be due to myosin, which does not extend into the H in this model. The M band is represented as a mass of material of unknown nature forming a bead on each actin myofilament midway between the Z bands. No provision is made for the N band in this model.

Vlés [138] interpreted the optical properties of striated muscle as indicating the presence of a greater density of protein material in the Q band than in the I. Hasselbach [59] has also advanced this hypothesis, basing his concepts on extraction procedures and on electron and polarization microscopy. Bennett [16] has shown with the interference microscope that the cross bands of unfixed relaxed rabbit psoas myofibrils are indeed characterized by differences in density. The interference data indicated that the amount of material per unit of length in the Z band peak was about 1.2 times that of the I band trough, whereas that of the Q and A peak was about twice that of the I and about 1.2 times that of the H minimum respectively. Engström [40] determined the ratio of mass per unit area of the A band to the I in *Chironomus* muscle and found it to average about 1.65:1. These ratios are most certainly too low, however, because the resolving limitations of the light microscope cause a fraction of the light traversing a certain band to be projected into the image of an adjacent band, leading to error in the direction of reduction of the amplitude of the peaks and valleys of retardation or absorption produced by the differences in density in the respective bands. Hasselbach [59] has concluded that the concentration of protein is 21



TEXT FIGURE B. MODEL OF THE SARCOMERE after Hanson and Huxley [54]. Two sets of myofilaments are represented. Thin actin filaments (a) extend from the Z band as far as the H, interdigitating with myosin filaments (m), which are confined to A, including the H. No M or N bands are represented, nor are cross filaments between myofilaments indicated.

per cent in the A band and 5 per cent in the I. Bennett and Porter [13] made tracings of osmium-fixed, sectioned avian muscle and showed that the sequence of cross-band densities observed in fresh myofibrils holds also for the osmium-fixed ones, but they did not determine any mass or density ratios. The electron micrographs of Hall, Jakus and Schmitt [51], Perry [101], Perry and Horne [103], and Draper and Hodge [35] sustain this general thesis.

Vlés [138] went further in his hypothesis, postulating that the striated muscle fibril was characterized by a continuous component running through all bands. He superimposed on this continuous structure at the sites of the A bands some additional materials characteristic of those bands, marked by birefringence and specific light absorption. Hasselbach [59], Hanson and Huxley [53], and Huxley [71, 72] have brought forth evidence that actin may be an important component forming continuous structures through all bands and that myosin may be localized in the A or Q bands in relaxed myofibrils, except perhaps in the M. This may provide a reasonable basis for accounting for the greater mass per unit of length characterizing the A band, as compared to the I. There is as yet no satisfactory evidence for conclusions regarding the localization of the tropomyosin or of the bound nucleotide in any particular cross bands of the myofibril. Meigs [89] reported the A band to absorb strongly in the ultraviolet, whereas Caspersson and Thorell [30] have reported the I band to contain substances absorbing strongly in the ultraviolet. These conflicting studies leave the localization of the nucleotides in doubt. Scott [127] demonstrated by microincineration methods a concentration of mineral material in the A bands and a lesser concentration in the Z. Whereas Engström [39] has reported the main concentration of mineral substances to be in the I, it is possible that there may be an error in band identification in this work, since it depends on the doubtful localization of ultraviolet-absorbing substances reported by Caspersson and Thorell [30].

The strong birefringence of the A band of muscle was first reported by Brücke [23], who also noted the lesser birefringence of the Z and N bands. Brücke's interpretation of the polarization optical properties of the myofibril was remarkably sound and penetrating; he held that these properties betokened the presence in the A band of asymmetric submicroscopic particles oriented with their long axes parallel to the axis of the fibril. Little has been added since to this concept except the postulate that these birefringent particles might be myosin molecules [59, 95, 96].

No tempting chemical explanation for birefringence of N or Z has been offered; Schmidt [119, 120] showed that the I band displayed a weak birefringence in balsam. However it is not yet known to what extent intrinsic birefringence may characterize the I band, as that observed in balsam might be largely form birefringence. The birefringence of the muscle fiber, most of which resides in the A band of the myofibril, has a large intrinsic component and has been thought to resemble that of oriented myosin threads [96].[5]

Rather striking variations from the usual banding patterns of relaxed muscle are often encountered. Bowman [20] noted this variation, though he did not observe all the variants which have been described since. He noted that during contraction the cross band periodicity lessened and the fiber increased in diameter. More detailed descriptions of contraction were offered by Dwight [37], who pictured a sequence of changes from well-relaxed sarcomeres to strongly contracted ones. The latter show a relatively broad dense band in the position of the Z and very little if any differentiated banding between. The broad dense peaks at the Z have since been observed many times in contracted myofibrils and have come to be known as contraction bands. Examples of typical contraction bands are depicted in Figures 180, 181, and 182. Figure 182 is from Engelmann's careful analytical study of the cytology of muscle contraction [38], and exhibits the appearance of relaxed and contracted sarcomeres with transitional stages as seen in ordinary and in polarized light. Relaxed sarcomeres are represented at the top and contracted ones at the bottom. Note how the sarcomeres are represented as shortening and broadening with contraction. As contraction progresses, the I band is represented as becoming shorter and shorter until the margin of the A band abuts onto the Z; with further shortening the A band broadens and becomes more dense. The zone of maximal birefringence in the sarcomere retains its position midway between the Z bands in all stages of contraction and relaxation. Engelmann's representation of the band changes with contraction remains valid today, except for two minor modifications. First, whereas he represents the Z band as being more dense than the Q or A in relaxed muscle, interference microscopy [16] and densitometry of electron micrographs [13] have shown that the Z band peak is in fact less dense than that of the Q or A. Second, Engelmann portrays very sharp transitions between successive bands in the sarcomeres, in accordance with the "square wave hypothesis," whereas in fact the transitions between the bands are more gradual than depicted (see p. 355).

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[5] Readers desiring to acquaint themselves with the significance of intrinsic and form birefringence may consult [12].

The sequences of contraction described by Dwight [37], Engelmann [38], and others involve a change in the relative densities of the cross bands. Whereas the A band is most dense in relaxed muscle, the Z is the most dense in contracted. This phenomenon has been termed a "reversal of striation" by Jordan [75]. However Engelmann and Jordan both point out that there is no reversal or change with contraction of the anisotropic segment (A band) as seen with polarized light.

But the changes in banding with contraction discussed above still do not portray all the variants seen in banding. Figure 180 reproduces a sequence of sarcomeres of *Hydrophilus* as depicted by Cajal [28]. This sequence shows sarcomeres with typical contraction bands, a segment of myofibril with little or no evidence of banding, a segment without Z bands, some myofibrils without H bands, others showing H bands but no N, sarcomeres with N bands, and finally some showing M bands in addition to all the others. Virtually all these sarcomere-banding types have been encountered in electron micrographs of avian muscle by Bennett and Porter [13], who presented densitometric tracings along several of them.

The type of sarcomere banding represented in Cajal's [28] Figure 49 (see Fig. 180 of this paper) is often said to be associated with slightly stretched, relaxed myofibrils. No satisfactory physiological correlation has been offered for the types of banding indicated in Cajal's Figure 47 by C and B (Fig. 180). Bennett and Porter [13] have suggested that these may characterize a phase in the relaxation of muscle.

At present, however, our biochemical and physiological understanding of the cross bands of muscle and of the variations and changes encountered in them is unsatisfactory. If suggestions that the A band is characterized by myosin are correct, there is no satisfactory explanation in chemical terms of the Z, M, and H bands.

That the myofibrils are comprised in turn of finer, filamentous, oriented elements was ascertained by Kölliker in 1888 [81], whose illustration depicting this observation is reproduced here as Figure 181. Kölliker describes this in the following explicit words (p. 694):

> "Ganz andere Art sind Beobachtungen bei *Melolontha,*[6] die auf eine Zusammensetzung der Fibrillen aus noch feineren Fäserchen hin-

[6] *Melolontha* is a genus of beetle.

> weisen. Einmal sah ich an frischen Muskeln stärkere Fibrillen, die zwischen je zwei Z mit Linse 3,0 mm von Zeiss und dem apochromatischen Oc. 12 deutlich und klar eine regelmässige dichte Strichelung zeigten (Fig. 2 A_1), von deren Vorhandensein auch Dr. O. Schultze sich überzeugte. Zweitens fand ich ein paar Mal das Glied Q am Ende von Fibrillen wie pinselförmig ausgefasert (Fig. 2A_2) . . ." [See Fig. 181, this paper.]

Kölliker was cautious in attributing general significance to these observations, remarking that they merely pointed to the possibility that the myofibrils were composed of bundles of still finer filamentary elements. It is clear, however, that Kölliker did distinguish the presence of the structures now known as myofilaments. Retzius [115] and Jordan [75] also realized that the myofibrils were composed of smaller fibrillar units. Hall, Jakus, and Schmitt [51] described these filaments clearly as they appear in electron micrographs of isolated myofibrils. These have come to be called myofilaments. They can indeed be distinguished with the light microscope if frayed out so that individual filaments are separated by resolvable distances, as Kölliker showed in his Figure 2 A_2 (Fig. 181) and as the late Dr. Paul Ralph demonstrated to me on several occasions.

Since Hall, Jakus, and Schmitt's important paper of 1946 [51], the myofilaments have been demonstrated many times in electron micrographs, amply confirming the correctness of Kölliker's concept [13, 35, 41, 65, 66, 71]. Hall, Jakus, and Schmitt [51] noted that the filaments were not uniform throughout their length, but presented a series of beadlike enlargements occurring every few hundred A. Draper and Hodge [35] demonstrated these enlargements as a regular periodicity occurring every 400–500 A along each myofilament, with the nodosities in phase with each other across the myofibril. Hall, Jakus, and Schmitt showed that the filaments within the myofibrils were dimensionally similar to the "myosin molecules" isolated from muscle in alkaline KCl solution by the method of von Muralt and Edsall [94, 95].

A second type of periodicity associated with the myofilaments was reported in 1950 by Hoffmann-Berling and Kausche [66]. These authors studied isolated fragmented myofibrils of frogs with the electron microscope and noted that the myofilaments were connected with each other by fine filamentary cross bridges occurring every 220–250 A along the length of the sarcomere. These cross bridges were later reported in sectioned avian muscle by Bennett and Porter [13] and are shown in a longitudinal section of contracted muscle in Figure 186. The cross-bridge periodicity is here shortened to less than 200 A. The cross filaments have been demonstrated in cross sections of insect myofibrils by Hodge [65], one of whose elegant micrographs is shown in Figure 183 of this paper. The cross bridges are seen binding the myofilaments together in an hexagonal array, the longitudinal myofilaments being spaced 300–400 A apart, center to center. Hodge states that these layers or planes of cross bridges repeat themselves longitudinally every 250 A in the A, I, and Z bands of the sarcomere but not in the H or M bands. This axial cross-bridge periodicity of 250 A has not been reconciled in a satisfactory manner with the 450 A beaded periodicity mentioned earlier.

The hexagonal pattern formed by the longitudinal myofilaments as seen in cross section was first reported by Morgan, Rozsa, Szent-Györgyi, and Wyckoff [93] and has since been confirmed by a number of workers [13, 64, 65, 71, 72]. As initially reported by Morgan and his co-workers, the myofilaments were envisioned as being arranged in a simple hexagonal close packing. This view was reflected by Bennett and Porter. Huxley, however, proposed a more elaborate view of the cross-sectional packing. On the basis of X-ray diffraction patterns [72, 54] and electron micrographs [71] of rabbit psoas muscle, he postulated the myofibril to contain two kinds of myofilaments. Cross sections of a myofibril through the A band (outside the H or M) are represented as showing both types of filaments (see Fig. 184). The large black dots are deemed to be filaments of "myosin," about 110 A in diameter, arranged in hexagonal array 200–300 A apart in the sectioned material. Between these filaments are more delicate structures which Huxley regards as consisting of "actin" filaments, each located at the center of an equilateral triangle, the apices of which are defined by the hexagonally packed "myosin" filaments. Only large "myosin" filaments are found in the H band, where they are reported as being thicker (140 A) than in the A. Only slender "actin" filaments are described by Huxley in the I band, where no regular hexagonal orientation was detected.

On the basis of these studies, Hanson and Huxley [54] have proposed a model of the striped muscle sarcomere on which a theory of contraction has been elaborated. This model postulates two types of filaments, one of myosin and the other of actin. Through the courtesy of Dr. Huxley, a diagram of this model of myofibrillar structure has been generously provided for this paper (Text Fig. B). The relatively coarse "myosin" filaments are postulated to run longitudinally through the A band, including the H and M, but not to intrude into the I or Z in relaxed muscle. The more slender "actin" filaments are envisioned as anchored in the Z band and extending thence into the sarcomere on each side, through the I and a portion of the A, but stopping short of the H band. The H band is thus represented as a portion of the myofibril which contains "myosin" filaments but not "actin," whereas the I band is said to contain "actin" but not "myosin" filaments. The Z, N, and M bands are not accounted for in this hypothesis. During contraction, an interaction between the "actin" and "myosin" filaments is postulated. This interaction is characterized "by a process in which actin filaments slide out of or into the parallel array of myosin filaments in the A bands." As shortening proceeds, the actin filaments are deemed to fold up in the A band. "This folding continues after the I bands have been fully retracted. The myosin filaments remain at constant length until forced to shorten by excessive contraction of the sarcomeres."

Huxley and Hanson's model neither accounts for nor recognizes the cross bridges of Hoffmann-Berling and Kausche [66] spaced axially every 250 A, demonstrated so convincingly by Hodge [65], and shown in Figure 183. Hodge's pictures show no structures in the centers of the equilateral triangles formed by the large myofilaments in the position where Huxley postulates the actin filaments to reside. This discrepancy cannot be dismissed lightly on the grounds of some hypothetical fundamental difference between vertebrate and invertebrate muscle. The cross bridges have been reported in muscle of frog [66], domestic fowl [13] (Figs. 13, 14), grasshopper [14], fly [65] (Fig. 11), and giant diving beetle (Fig. 21). Bennett and Porter's [13] cross sections of bird muscle, one of which is reproduced in Figure 185, are not inferior in resolution to Huxley's and show no second set of filaments. The low angle X-ray diffraction patterns cited by Huxley [72] in support of his concept for a second set of filaments were published by Hanson and Huxley [54]. They contain only two or three lines and hence provide very little information. Moreover, Huxley [72] states that several interpretations can be given to the results and that certain unstated assumptions underlie the interpretation he chose. Thus one cannot regard the X-ray evidence for the Huxley model as compelling, and the electron micrographs emerge as the main support for the model. Huxley has courteously provided the print for Figure 184 as the best of the electron micrograph prints of rabbit psoas muscle supporting his hypothesis.

It is of interest to compare Huxley's model of the sarcomere with that of Hodge [65], who has generously supplied the print for Figure 183 and the diagram for Text Figure A for this paper. Hodge and Huxley agree with Hasselbach [59] in basing their models on the view that myosin is localized in the A band and actin in both the A and I bands, but they differ in their postulates as to the disposition of myosin and actin within the A band. Hodge postulates a single set of axial myofilaments bound together in hexagonal array in the A, I, and Z bands by the cross bridges mentioned above. These bridges are believed to be absent from the H and M. Each myofilament is conceived as running continuously through all bands but as differing in its structure as it traverses this band or that. In the A band (Fig. 183) Hodge states that the myofilaments display "a 'tubular' or compound cross section," with "lightly stained medullary region about 40 A in diameter . . . surrounded by a thin dense cortex," leading to an over-all myofilament diameter of 100–120 A. The light "core or medulla of the myofilament appears to be structurally continuous with the filaments of the H, I, and Z bands." The cross-bridging filaments are measured to be about 40–60 A in thickness. Hodge represents the myofilaments in the H band as lacking the dense cortex characterizing his model of the A; the M band is conceived as a row of fusiform beads or thickenings, adorning each filament (Text Fig. A).

Hodge and Huxley also differ in their views of the nature of the changes occurring in contraction. Hodge embraces the conclusion of Jordan [75] that there is a true migration of "A substance" from the A into the I band, but at the same time he confirms Engelmann's observation that the anisotropic band does not shift position during contraction [38]. Huxley and Hanson conceive of the Z band as being drawn up into contact with the A during contraction as the "actin" filaments interact with the "myosin" filaments. This view is endorsed by the studies of Huxley and Niedergerke [69], using the interference microscope.

Further quantitative work with the polarizing and interference microscope is necessary if a satisfactory understanding is to be achieved of the changes in striation with contraction, insofar as they can be resolved with the light microscope. Our knowledge of the chemical nature of the cross filaments and of the Z, H, N, and M bands is similarly unsatisfactory. It seems evident that our comprehension of the structure of the striated muscle myofibril is not yet sufficient to lead to acceptance of any detailed model or concept of contraction.

7. *Sarcoplasm*

Sarcoplasm—the content of the sarcolemma outside the nuclei and the myofibrils—comprises 30 to 70 per cent of the volume of the muscle fiber; yet it has received relatively little attention in recent years and is seldom considered in theories of muscle contraction. It has often been ignored or dismissed as a mere unorganized proteinaceous medium providing metabolic support for the myofibrils. Although it is reasonable to assign an important metabolic role to sarcoplasm, it is now evident that it is far from unorganized, and some of its structural features suggest that in it may be localized important physiological functions, such as conduction of impulses within the fiber.

Yet fifty to seventy-five years ago the sarcoplasm and its contents formed the object of much study, and a considerable degree of organization was recognized in it. Though various components were characterized, classified, and named, most of this literature seems to have been ignored in recent articles. Bennett and Porter [13] revived interest in the organization of sarcoplasm by demonstrating with the electron microscope the correctness of some of the concepts of sarcoplasmic structure prevalent at the turn of the century, and the time is now ripe for making further progress in our understanding of the extrafibrillar components of the fiber.

Schwann [125] in 1839 described and pictured granules in muscle cells which were distinct from the nucleus and from the cross-striped fibrillar material. Two years later Henle [63] mentioned fine granular material between the fibrils of striated muscle and noted these granules to be resistant to acetic acid. The first observation reporting an organization of interfibrillar material in a pattern related to the cross bands of the myofibrils appears to be that of Dobie [34], who, in 1849, studied individual vertebrate myofibrils which had been carefully dissected apart; he noted as the fibrils were separated that a very delicate membrane-like material could be seen stretched out between the separated fibrils. In some instances this interfibrillar material was seen in stripes corresponding to the cross bands. One of Dobie's drawings illustrating this phenomenon is reproduced in Figure 179. In 1853 Aubert [7] pictured granules between the fibrils of insect flight muscle. This may well be the earliest representation of objects definitely recognizable as the structures now known as mitochondria.

Kölliker [79] in 1857 described and classified two formed components of sarcoplasm. Between the fibrils he recognized fat droplets and "interstitial granules," which Retzius [115] later called sarcosomes and which Regaud [112] and Regaud and Favre [113] first recognized as true mitochondria. Many years after Kölliker, Arnold [1–4] described a third granular component of sarcoplasm—the glycogen granule. The fat droplets, the mitochondria or sarcosomes, and the glycogen particles remain today the three principal types of particles to be seen with the light microscope in the sarcoplasm.

Harman [56, 77] and his co-workers have recently attempted to introduce a new classification of sarcoplasmic organelles. They choose to use the word "cytochondria," which they credit to Opie, who defined the term as applying to all the cytoplasmic bodies which are recognizable. The term is applied to essential constituents of the cytoplasm, including those that stain or fail to stain as mitochondria. Kitiyakara and Harman [77] then go on to say: "The cytochondria of skeletal muscle are comprised, therefore, of the mitochondria and the small particles of Floegel, which later will be designated specifically as sarcosomes. Fat granules or liposomes may also be included under cytochondria, and, perhaps, contain the structures described as Golgi bodies in other types of cell." The usage of the word "sarcosome," chosen by Harman and co-workers, seems to be unnecessarily confusing. The word was introduced by Retzius [115] in 1890, who applied it to the interstitial granules of Kölliker. Regaud [112], Bullard [26, 27], and many others have shown these structures to be mitochondria, and the term "sarcosome" is well entrenched in the literature as a term synonymous with the mitochondria of muscle. Harman and his group use the word "mitochondria" quite properly, in accordance with established usage. The structures they describe as "sarcosomes," however, have the following characteristics [77, p. 560]: size of 0.1 to 0.3 μ, round or elliptical shape, strong "refringence," strong osmiophilia, slight ability to bind aniline dyes, absent osmotic swelling, complete solubility in organic solvents, and arrangement transverse to myofibrils. Harman's Figure 2 [56] shows his "sarcosomes" lying generally opposite the Z and I bands; his mitochondria are found alongside any band.

From these characteristics one might be led to believe that the structures Harman refers to as "sarcosomes" could well conform to the fat droplets described so often in the sarcoplasm. The so-called "sarcosomes" in Harman's [56] Figure 2 correspond exactly in distribution and relative size to the fat droplets demonstrated with Scharlach R by Bullard [26] in the breast muscle of the pigeon (Bullard's Figs. 5, 6). The osmiophilia shown in Harman's Figure 2 and mentioned in his Table 1 is again consistent with the view that these particles are lipoid in nature. Yet Harman [56, p. 70] says, "From these analyses it is apparent that the sarcosomes contain considerable nucleic acid and are not identical with the neutral fat particles or liposomes." This statement is unconvincing, since the analyses referred to are of centrifuged fragments of these particles separated from his "cytochondrial" fraction. Harman gives no evidence that he studied the purity of the so-called sarcosome fraction used in this analysis. Nor does he give evidence that it was free of mitochondria, sarcoplasmic reticulum, matrix, or other components outside these lipoid-rich bodies which might contribute significantly to the nitrogen, lipide phosphorus, or pentose-nucleic acid values determined for the sample. He gives no values for the neutral fat content of the sample. Harman refers to a paper by Flögel [45], stating [77, p. 554], "Later Floegel found a second small isodiametric granule which he distinguished from the Koelliker granule because of differences in size and shape." Careful reading of Flögel's paper reveals no justification for his claim that his granules were distinct from Kölliker's interstitial granules. Flögel's granules are well within the ranges of size and shape of the mitochondria of muscle. They occupy positions in rows across the fiber at levels corresponding to the junction between the A and I bands, on each side of the Z. This is a very common location for mitochondria, as was well brought out by Retzius [115], who called them "sarcosomes." Thus one is led to the following view regarding the classification of the "cytochondria" of muscle as proposed by Harman and co-workers: Harman's "mitochondria" are true mitochondria and are the equivalent of the sarcosomes of Retzius and the interstitial granules of Kölliker. Harman's "sarcosomes" appear to correspond to the fat droplets of Bullard and Kölliker. The use of the term "sarcosome" as proposed by Harman should not be continued.

A meaning of the term "sarcosome" proposed by Kisch [76] is "to call all microscopic and submicroscopic inclusions in muscle plasma, sarcosomes except fat droplets, pigment granules and disintegration products of muscle fibrils." This definition is hardly satisfactory, since it would include glycogen, mitochondria, and elements of the sarcoplasmic reticulum. Kisch includes in his paper an interesting historical survey of concepts regarding granules in the sarcoplasm as they have developed over the last century and a half. His interpretation of the significance of some of the early work differs in some respects from that presented in the present text.

GLYCOGEN. So far as the published record appears, glycogen has not been recognized in electron micrographs of muscle fibers. Our concepts of glycogen distribution in muscle, therefore, depend on the light microscope studies of Mancini [87] and Arnold [1–4]. These investigators describe glycogen as occurring in granules in the sarcoplasm, located in transverse rows close to the junction of the A and I bands on each side of the Z. This corresponds to a location where mitochondria and transverse elements of the endoplasmic reticulum are also encountered.

LIPIDE INCLUSIONS. Although spheroidal fatty inclusions in striped muscle were recognized with the light microscope by Kölliker [79], a clear distinction between fat droplets and mitochondria awaited the important studies of Bullard [26, 27]. A noteworthy study by Greene [48] established the concept that the very large quantity of fat stored in muscle cells of salmon prior to their fast in upstream migration is to be regarded as fuel in storage and in no sense represents fatty degeneration. This fat is used up during the migration. Greene noted that during the feeding stage fat is deposited mainly in fat cells between the fibers, but at the beginning of the spawning migration the pink muscle fibers come to be loaded with numerous chains of very small liposomes. The dark muscle along the lateral line of the salmon accumulates an enormous load of intramuscular fat. Most of this is also used up during migration. The fine liposomes are depicted by Greene as being disposed in rows between the myofibrils without any special relation to the cross bands.

Bullard [26] and Knoll [78] found lipoid bodies to be particularly abundant in pigeon breast muscle, where Harman and Osborne [55], Harman [56], and Kitiyakara and Harman [77] found them but called them "sarcosomes." The confusion created by this unfortunate choice of terms has been dealt with above (p. 366–7).

MITOCHONDRIA. The discovery of the mitochondria of muscle long antedates the recognition of mitochondria as a formed component of cells in general. These mitochondria of muscle were originally termed the interstitial granules of Kölliker [79] or the sarcosomes of Retzius [115]. Regaud [112] and Regaud and Favre [113] postulated that these correspond to mitochondria. This viewpoint has been confirmed on biochemical grounds by Watanabe and Williams [140, 141], on morphological grounds by Bullard [26, 27], Bennett and Porter [13], Palade [98], and many others, and on cytochemical grounds by Wachstein and Meisel [139].

Morphologically, the individual mitochondria of muscle are built on the membrane and cristae plan of Palade [98]. Weinstein [145] has shown that in mitochondria of heart muscle the cristae are often tubular, resembling those in mitochondria of *Paramecium* as described by Sedar and Porter [128]. In many insect muscles, as in the flight muscles of *Hydrophilus piceus* (Fig. 173), the cristae mitochondriales appear as densely packed parallel lamellae.

The abundance and geometric disposition of mitochondria vary greatly in different sorts of muscle. In the white breast muscle of the domestic fowl, studied by Bennett and Porter [13], few mitochondria are to be seen. These are disposed irregularly in the sarcoplasm. In contrast, insect flight muscle contains very numerous and large mitochondria (Fig. 173), as does vertebrate heart muscle [145, 76]. Wachstein and Meisel [139] have emphasized that individual muscle fibers within a single muscle may vary greatly with respect to numbers of mitochondria contained within them. Some of the variants encountered in the arrangement of mitochondria within muscle fibers may be ascertained by studying the work of Kölliker [79, 81], Retzius [115], Bullard [26], and Thulin [135]. A representation of mitochondria distributed as they might be in a muscle fiber from the diaphragm of a mouse is shown in Figure 194. One encounters mitochondria frequently in rows or columns here and there, distributed longitudinally between myofibrils, and one also finds them between the sarcolemma and myofibrils, often in dense pads or masses of many tens or hundreds. Mitochondria are particularly abundant near motor nerve endings.

SARCOPLASMIC RETICULUM. Although Dobie [34] observed a structural arrangement in sarcoplasm related to the cross bands of muscle, more than a quarter of a century elapsed before attention was again focused on an organized component of the sarcoplasm. Thin [133, 134] in 1874 and 1876 impregnated muscle fibers with gold and observed between the fibrils a fine network comprised of transverse and longitudinal fibers. Retzius [114] in 1881 confirmed the observations of Thin and, on the basis of more elegant preparations, observed transverse elements of the reticulum at the levels of the Z, M, and N bands. He also noted longitudinal members of this network and suggested that the whole three-dimensional network might participate in the transmission of excitation within the muscle fiber. A series of papers dealing with the sarcoplasmic reticulum appeared during the next quarter-century, of which the most noteworthy are those of Melland [90], Marshall [88], Cajal [28], Retzius [115], and Veratti [137]. Melland noted that the elements of the reticulum were adherent to the inner surface of the sarcolemma. Cajal reported seeing the reticulum in living unfixed muscle (Fig. 192). Retzius saw that the reticulum was closely associated with sarcosomes (mitochondria) and often connected with myofibrils at the Z band (Fig. 177).

Veratti's [137] monumental paper of 1902 stands as the most definitive and comprehensive study of the sarcoplasmic reticulum existing in the literature and must be regarded as one of the classics of muscle structure. Veratti, a pupil of Golgi, made silver impregnations of the reticulum in a great variety of mature and immature vertebrate and invertebrate muscle and illustrated his findings with colored drawings of exquisite delicacy and beauty. Three of his pictures are reproduced in this paper (Figs. 187, 188, 190), although these reproductions do not do justice to the elegance of the originals. Veratti showed that the sarcoplasmic reticulum varies considerably in its arrangement from muscle to muscle. For example, in the newborn mouse and in embryo lizard Veratti found muscle in which the reticulum forms a rather irregular meshwork within the fiber. However, in most vertebrate and invertebrate muscle the transverse members of the reticulum are related to the cross bands in one way or another. Common sites for cross-band skeins are at the levels of the Z bands, the levels of the M bands, and levels close to the junction of the A and I bands. The longitudinal component of the reticulum is represented as pursuing crooked paths—an observation at variance with those of previous workers but recently confirmed with the electron microscope [15, 110]. Veratti noted that the reticulum made contact with the sarcolemma and with the myofibrils.

Heidenhain [62] represented the transverse component of the sarcoplasmic reticulum in his Figure 279. He realized that the reticulum connected with the Z bands, but he did not recognize the longitudinal component, nor did he understand fully the nature of the Z and M bands, which he represented as transverse membranes across the fiber, continuous through myofibril and sarcoplasm alike. Von Boga [19], Bennett and Porter [13], Bennett [15], and Porter [110] have shown that Heidenhain's model is incorrect in this detail, since the Z and M bands are confined to the myofibril. Connections between one myofibril and another are provided by elements of the sarcoplasmic reticulum, which is entirely different in structure from the Z band but which connects with it.

Von Boga [19] has contributed a noteworthy study of morphological manifestations of the sarcoplasmic reticulum as it may traverse extensive subsarcolemmal pads of sarcoplasm to the sarcolemma (Figs. 176, 189). His study indicates clearly that the geometrical arrangement of the extensions of the sarcoplasmic reticulum extending from myofibrils to sarcolemma is not appropriate for the transmission of tension.

Referring once more to Retzius' suggestion of 1881 [114] that the sarcoplasmic reticulum might participate in conduction of an excitatory impulse within the muscle fiber, it is of interest that there are many references in the literature to some sort of special connection between fibrillar elements in the motor end plate and the sarcoplasmic reticulum or the Z band, which—as has just been stated—is actually connected to the reticulum at each myofibril. Marshall [88], Gerlach [47], Bremer [22], Boeke [18], Foettinger [46], Thanhoffer [132], Rollet [116], Beams and Evans [11], and Tiegs [136] are among those who have reported observing such connections. One of Thanhoffer's illustrations is reproduced in Figure 191 of this review. However, most depictions of the motor nerve plate show no special connections between elements in the nerve terminal and organized sarcoplasmic units in the fiber. A satisfactory understanding of the nature and significance of these connections must await further work.

Recently Huxley and Taylor [70] have been able to produce localized contractions involving one sarcomere of a muscle fiber. The contractions extended a short distance into the fiber from the sarcolemma immediately adjacent to a stimulating microelectrode touching the sarcolemma at the level of a Z band. This was interpreted as evidence for a transverse conducting member within the fiber at the level of the Z.

A model of the muscle fiber showing relations of the sarcoplasmic reticulum is presented in Figure 194. The variant chosen is one similar to that seen in mammalian diaphragmatic muscle. The reticulum is shown as being comprised of membranes studded on the outside with dense particles and arranged in tubules and cisternae, like those of the endoplasmic reticulum [99, 109]. The reticulum is represented as making contact with the sarcolemma at the level of the Z band and as forming delicate, lacelike bracelets encircling the myofibrils close to the junction between the A and I, and in the mid-H region. Longitudinal elements of the reticulum are represented in tenuous, irregular skeins sending connections to the Z bands of the myofibrils. The reticulum comes into close relationship to the mitochondria. The grosser features of the distribution of the reticulum represented in the model are based largely on the work of Veratti. The finer details are taken from the work of Bennett [15], Porter [110], and Palade [99].

SARCOPLASMIC MATRIX. Kölliker [79] recognized a continuous phase in the sarcoplasm, composed of proteinaceous fluid. Bennett and Porter [13] spoke of this as the sarcoplasmic matrix. It is regarded as an unorganized solution, perhaps containing suspended particles, extending continuously around reticulum, mitochondria, lipide droplets, and myofibrils, and perhaps permeating the latter as well. It is regarded as containing the myogen proteins of muscle [9].

8. *Myotendinal Junction*

The sarcolemma is continuous over the muscle fiber at each end and along the sides. The manner in which the tension generated by the contracting muscle fiber is transmitted to the tendon has been a topic of controversy for many years. Since the limits of resolution of the light microscope had led to the erroneous conclusion that the Z band or telephragma was continuous across the fiber [62, p. 518], consideration has been given to the view that this hypothetical continuous structure might be mechanically stiff and strong, and transmit tension from the contracting myofibrils to the sarcolemma, whence the tension would be taken up by collagen fibers outside the fiber [50]. However, this view must be abandoned, since it has been demonstrated by the electron microscope that the Z bands are not continuous from myofibril to myofibril, but are connected to each other by delicate flexible strands of sarcoplasmic reticulum. Bowman [20] showed that the contractile portions of the fiber were but loosely attached to the sarcolemma (Fig. 175). Bennett and Porter [13] have shown that one myofibril can be displaced longitudinally with respect to a neighbor without distorting or damaging the delicate myofilaments in any way. This would seem to argue decisively against the existence of any stiff, strong, lateral structural membranes connecting myofibrils to one another and to the sarcolemma at the level of the Z. It does not, however, rule out the possibility of large numbers of weak valence forces binding myofibrils laterally to each other, as suggested by Barer [10]. Such forces have not been demonstrated but could reasonably be postulated, and might account for the tendency of the cross bands of neighboring myofibrils to line up in register with each other. Such forces in aggregate might play an appreciable role in transmitting tensile forces laterally to the sarcolemma.

The myotendinal junction is of particular interest with respect to the transmission of contractile tension. Bowman [20] noted a direct continuity between myofibrils and tendinal fibrils (Fig. 178). Schmidt [121] demonstrated a continuity of birefringent fibrils from contractile to tendinal elements. Von Boga [19], in his extensive study of insect muscle, showed details of the myotendinal junction very clearly (Figs. 176, 189). They show continuity of myo- and tendinal fibrils and in addition show delicate, gossamer-like skeins of reticulum bridging broad sarcoplasmic pads between myofibrils and sarcolemma. These skeins scarcely comprise a connection suitable for transmitting tension to the sarcolemma. Porter [108] reported in abstract form that electron micrographs of myotendinal junctions in embryonic myoblasts of *Ambystoma* larvae show myofibrils to be connected to tendinal fibrils only through longitudinal arrays of the fine fibrillar elements of the sarcoplasmic ground substance. Clearly, additional study of the myotendinal junction is needed before its fine structure can be understood adequately.

9. *Conclusion*

The analytical approach has shown us where myosin and actin are located in the muscle cell and has informed us that the mitochondria of muscle behave biochemically like mitochondria of other tissues. But many morphological features of muscle still remain unexplained chemically and not understood physiologically. It is not yet time to formulate any very detailed theory of muscular contraction.

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13. *Cell Transformation and Differentiation in Regenerating Striated Muscle* [1]

BY GABRIEL C. GODMAN

1. Introduction

IN PHYSIOLOGICAL CONDITIONS adult tissues usually reveal only a few of their potencies, for it is usually held that with specialization cells may lose much or all of their earlier capacities for proliferative growth. But the "natural experiments" of disease and injury which are studied in pathology bring to expression latent powers of the specialized cells of the adult organism for proliferation and transformation which would not otherwise be exhibited. These are especially manifested in regeneration and in tumor growth.

The ability of organisms to regenerate lost parts or members diminishes in phylogenetic evolution as in ontogeny, and in higher vertebrates it is limited to the repair of individual tissues. Tissue repair is the replacement of lost cells by new ones of any type. If the new cells are specialized cells, similar to the ones they replace, the process is called regeneration. Regeneration necessarily involves proliferation and differentiation of cells. "Physiological" regeneration is constantly occurring from "cambium" layers of "intermitotic" cells [24], such as those of the Malpighian strata of the skin, or the hemocytoblasts and intermediate cell generations of the haematopoietic organs. Regeneration consequent on tissue damage or loss may, in addition, activate the more specialized cells of an affected tissue, such as those of liver, kidney, or glands. These cells, because they do not usually divide, but can be stimulated to do so, have been called "reverting postmitotic" cells [24].

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Compensatory and pathological hyperplasias are special instances of the general capacity for regeneration.

It is assumed that with greater specialization cells lose the means of dividing and hence of regeneration (the "fixed postmitotic" cells). Such cells are the neurons, the retinal rods and cones and the granulocytes. Other highly differentiated cells, however, retain great latent proliferative abilities which, when exercised, coincide with a loss of some or all of the specialized attributes of the differentiated state. This simplification or disappearance of specialized structure and function is especially obvious in tissue culture, and has often been referred to as de-differentiation. Complete de-differentiation, however, has been taken to mean more than a loss of special structures and functions, but a reversion to a more general embryonic state. In de-differentiation, cells are supposed to gain "potential functions"—that is, the ability to redifferentiate into other quite different types of cells.

Cell morphology alone is of little help in deciding, in any particular case, whether de-differentiation has occurred, with its loss of type specificity, or whether there has been some less drastic change entailing merely a loss of some visible equipment. That is why, when confronted with cell changes, it would be important to know, at any stage, not only the appearance of the cell, but something of its chemical constitution, its functional activity or behavior, and if possible its prospects for further development (i.e. its prospective potencies). The cells of adult higher vertebrates are presumably incapable of true de-differentiation, but partial loss of differentiation with the acquisition of a strictly limited freedom of transformation may occur. P. Weiss [12, 114] has applied the term "modulation" to such cell transformations in which there is an apparent loss of differentiated structure and a change of behavior, but which he assumes are ultimately reversible and involve no changes in potency or type. Modulations, in this usage, would be simply "the variety of expressions that can be assumed by the cell on any level of differentiation" [114].

Regeneration, then, subsumes a temporary loss of the differentiated state accompanying cell multiplication, which is followed by cellular re-differentiation and histological reorganization. Cytodifferentiation is the progressive specialization, from a more homogeneous state, of those physical and chemical structures which enable a cell to function in its specific way. As the opposite of de-differentiation, it also implies, as the process of differentiation progresses, a loss of the ability to enter other pathways of development.

Sometimes, in response to certain morbid stimuli, or in unusual environments, a mature, fully differentiated tissue of one kind is transformed into a fully differentiated tissue of another kind, a change which we know as metaplasia. The conversion of various glandular mucosae into keratinizing stratified squamous epithelium, such as takes place in vitamin A deficiency, or in the endometrium after hyperestrinism [97], or the reverse process, which occurs on the addition of vitamin A to tissue cultures of squamous epithelium [35]; the development of heterotopic bone in fibrous connective tissue, or the reverse, are well-known instances of metaplasia. Since metaplastic changes occur only in proliferating cells, they really represent a form of regeneration with atypical re-differentiation [118].

These themes of proliferation, partial de-differentiation and modulation, redifferentiation, and metaplasia are illustrated in the example provided by the regeneration of the mature striated skeletal muscle of mammals, which will be described.

2. *On the Regeneration of Mammalian Striped Muscle*

In spite of a considerable amount of literature on striated muscle regeneration [see 2, 8, 45, 79, 80 for more recent contributions] it is still commonly held that "the regenerative capacity of the striated muscular tissue of vertebrates is usually insignificant" [77] or "restricted in degree" because of its high degree of differentiation [16]. It is, of course, the usual experience that the end result of most traumatic damage to a muscle, which includes its stroma, is repair by fibrosis or scarring, rather than effective regeneration. But this is an "accidental" mechanical result ensuing from the collapse of a guiding stromal framework and the disoriented overgrowth of fibrous tissue prior to the onset of muscle regrowth, which is then disturbed. In higher vertebrates there is no agency such as the nervous system in lower organisms (including urodeles) to influence an orderly organotypic regeneration in the absence of a pre-existing frame. Consequently, each cell type grows partly in competition with others, and in any case, in independent rather than integrated patterns. The production of scar tissue after traumata to muscle does not show that striated muscle cannot proliferate effectively. The tissue structure can be reproduced only when an organized scaffold of regular pattern is left behind to permit regrowth of its various components in particular lines and relationships. Such a scaffold can be provided in muscle when the sarcolemmal or the endomysial sheaths remain relatively undisturbed. This situation is in fact encountered after some toxic infections of muscle in which the muscle proper, particularly the contractile substance, is destroyed. To some extent it may be produced experimentally according to the method of Le Gros Clark and Blomfield [64] by ligating both branches of the main nutrient artery to the anterior tibial muscle of the rabbit. This loss of blood supply causes infarction of most of the distal two-thirds of the muscle. The proximal one-third and a narrow distal sector are preserved by small collateral vessels which enter at the muscle origin and insertion, and the fibers of this viable muscle are the chief source of the subsequent regeneration.

EVENTS IN THE REGENERATION OF THE ISCHAEMIC INFARCT OF MUSCLE. In the anemic part of the muscle the fibers undergo, in effect, protoplasmic clotting and clot retraction. At the surface of the infarcted area, the sound parts of the fibers, abruptly demarcated from the retraction caps of the injured segments, contract away from them, exposing partly empty tubular spaces encased by residual sheaths of preserved sarcolemma, or more usually fibroblastic endomysium, containing fragments of injured muscle. This interzone or border region with its tubular sheaths, between the viable and the necrotic tissue, is the stage upon which the subsequent events are unfolded. By the fourth day after ligation these sheaths have come to enclose a mixture of degenerated muscle fragments, various single cells, and regenerating sprouts, and were therefore first described as "muscle-cell sacs" or tubes (Muskelzellenschläuche) by Waldeyer [110]. Within the fully-developed tube of the fifth and sixth days, the most numerous elements, toward the necrotic side, are isolated mono- or binucleate cells, which closely beset the degenerated fragments and fibers and appear to be phagocytosing them. At the tube periphery, more closely applied to the inner aspects of the sheath, there are apparently single fusiform elements and lengths of slender ribbon-like sprouts or slips (Figs. 195, 196). Entering the tubes from the sound, vascularized muscle are the tapered distal parts of long wider ribbons; these are young regenerating elements which have originated from the ends of the still intact fibers, and are growing along the free space of the open tubes supported on the linear sarcolemmal or endomysial scaffolds. These wide and narrower plasmodial ribbons of regenerating muscle may be called sarcoblasts. Other plasmodial elements, such as detached muscle "buds" or apparently detached straps may also be found in the tubes (Fig. 199). After the fifth day degenerated fragments of muscle, cut off from relationship with the cell tubes by displacement or stromal collapse and overgrowth, are found surrounded by multinucleate giant cells.

In the reconstitution of the devitalized muscular tissue, the ingress of the sarcoblasts follows closely upon the clearance of the necrotic mass by phagocytic activity. At the end of a week young sarcoblasts have come to fill the extant tubes, their ingrowth guided by the endomysial planes. Proliferating fibroblasts from the surviving endomysium of the interzone gradually penetrate around the columns of the necrotic fibers of the infarcted area like advancing sleeves and replace the necrotic stroma of these areas, thus forming new sheaths for some distance about the necrotic fibers. As the necrotic material is removed by phagocytosis, the sarcoblasts grow into the new tubes, and in this way the pattern of the muscle is largely preserved in the zones which are repaired. The depths of the necrotic part are not regenerated, and fibrosis and atrophy of the muscle is the rule. But in the regenerated areas restitution is fairly advanced by the fourth or fifth week after infarction.

REGENERATION AFTER OTHER KINDS OF INJURY. The same events occurring in the process of regeneration after ischaemic infarction can also be discerned after other injuries which produce large areas of necrosis affecting primarily or solely the muscle fibers. After "Zenker's degeneration" following numerous infective diseases [39, 79], after infection by certain viruses (Coxsackie group and some neurotropic viruses [46, 95]), after intoxication by plasmocide [55], in vitamin E deficiency [85], after heat coagulation [2], gentle crushing [63], or freezing, cell tubes are formed and the regeneration of their contents may be quite complete. Differences in numbers and perhaps in the origins of the various elements which take part in repair of these lesions depend upon differences in the extent of damage sustained. With some derangement of architecture, as in crushing and freezing for example, intratubular giant cells are far more prominent; in the regeneration after murine Coxsackie virus disease, regeneration is more rapid and complete, and reactive cells are fewer. With allowance for species differences and for variations resulting from differences in kind of injury, however, the course of regeneration in all of these lesions is basically similar to that described in the healing of the infarct, which may therefore be used as a prototype.

REGENERATION OF SKELETAL MUSCLE IN VITRO. It is thus evident that mature striated skeletal muscle, highly differentiated though it be, is capable of active regeneration and re-differentiation. Its intrinsic potencies for proliferative growth and transformation are perhaps best illustrated in tissue culture, which is only a special case of regeneration or wound repair. Myogenesis from embryonic muscle tissue has been repeatedly studied in culture since 1916 [15, 19, 28, 41, 67, 70, 75]. Pogogeff and Murray [89] have shown that adult mammalian skeletal muscle can be grown in tissue culture. When mature skeletal muscle of rat is explanted, there first emerge "fibroblasts," usually followed, in our experience, by recurrent showers of large, rounded refractile cells, and after a lag period of seven to fifteen days by true sarcoblastic ribbons (Fig. 201). Pogogeff and Murray drew attention to this long latent period before muscle outgrowth as a factor permitting fibroplasia to gain an early predominance. The sarcoblasts may be continuous with the fibers of the explant, or may occur as detached straps. Isolated mono- or binucleate fusiform cells, apparently of muscle origin, and round or racquet-shaped multinucleate buds or giant cells are also encountered (Fig. 200). Kikuchi [60] and Pogogeff and Murray [89] described similar cell forms in cultures of mature mammalian muscle. All of the same cell types are met with *in vivo* in the regenerating rabbit muscle infarct and in other muscle lesions. It is instructive to investigate the origins, nature, and fate of each cell type in the lesions, in the light of the information provided by tissue culture, and with the additional aid of histochemical methods.

3. The Isolated Cells Occurring in Regenerating Lesions of Muscle

THE FREE CELLS IN VIVO. The free single cells within the sarcolemmal or endomysial tubes are of two kinds: (1) peripheral fusiform elements usually, but not invariably, applied against the inner wall of the tube; and (2) the more numerous, large, round cells with abundant cytoplasm (Figs. 195–199). The former have elongate nuclei, large nucleoli, and amphophilic or basophilic cytoplasm with filamentous polar extensions. The round cells have one or two spherical or indented darkly staining nuclei with one to four granular karyosomes, and granular acidophilic cytoplasm. They multiply by frequent mitoses. They are found clustered about degenerated muscle masses, with which they are in close relation, or they may lie in the interior or central parts of the tube lumens, toward the necrotic side (Figs. 195–197). Cells with features intermediate between these types are also to be seen in the tubes.

It would appear at first, as indeed some authors have assumed [39, 63, 80, 88], that most or all of the free cells of the tube are immigrated mononuclear (histiocytic) cells of the inflammatory exudate, and presumably either of hematogenous or connective tissue origin. But many appearances in the histologic sections suggest that both round and fusiform cells can sometimes be set free from partly damaged muscle fiber segments. These cells collect in clusters at the ends or peripheries of muscle fragments with which they are in close relation (Fig. 198). Their cytoplasm may be continuous with the sarcoplasm of the injured muscle, and often shows residual traces of striation or bundles of naked myofibrils partly contained within it. Of course, such histologic pictures, however suggestive, do not reveal the origin of the cells, or which of them are entering phagocytes and which derived *in loco*. Tubes with intact sarcolemmal casings are sometimes found containing isolated cells at a time (fourth day) when relatively few of them are present in the perimysial interstitium as compared with the tubes. Were all the cells coming from the perivascular zones to the tubes via the interstitium the concentration gradient might be expected to be the reverse in the third and fourth day lesions.

Within the superficial layers of the infarcted area many of the affected muscle fibers are not dead but have retained intact nuclei and sarcoplasm; only the contractile substance of these fibers is necrotic. This regressive change has been called "dissociative" degeneration [8, 88]. The surviving nuclei and sarcoplasm are often increased, and a few at least would appear to make their way out of the devitalized residue as single cells. Indeed, the breaking up of muscle fibers into isolated cell elements has been repeatedly described in the older literature [26, 109] and more recently [2, 8, 88, 98], but the nature and subsequent fate of the cells have remained in doubt. The idea of their transformation to other cell types is difficult for pathologists to accept. Forbus [40], Pfühl [88], and Betz [8] made attempts to clarify the nature of the isolated cells and the symplastic elements of the tubes in experimental lesions with the use of vital dyes. Forbus found that in animals which had been saturated with intravital dye prior to necrotizing injury, some cells within the muscle tube contained dye and were therefore presumably macrophagic invaders, while the others, either of muscle origin or daughter cells of the phagocytes, did not store dye. With continued administration of vital dyes, Forbus found that most free cells, including those presumably derived from muscle, were also capable of some arthrocytosis. Like Betz, we have found in the regenerating infarcts of intravitally dyed animals that many but not all of the free round cells store dye particles and that the sarcoblasts, muscle buds, and most of the fusiform cells do not. It must be agreed with Forbus that intravital staining does not conclusively reveal the origin of all of these cells. Evidence of the phagocytic nature of the free round cells of the tubes is everywhere obvious in the lesions, and ingested red blood corpuscles and hyaline necrotic muscle particles can often be found in the cytoplasm.

THE FREE ROUND CELLS IN VITRO. Inferences concerning the origin and nature of the free cells obtained from static histologic preparations can be checked by observation of changes in regenerating muscle divorced from blood and nerve supply, such as is possible in tissue culture. The large, round, refractile cells of the rat muscle cultures, which issue forth in bursts, have the characteristics which we associate with amoeboid wandering cells or macrophages *in vitro*. They are actively motile, possess undulating membranes, and are always closely applied to the glass interface or cluster on the surfaces of fibers and particles (Fig. 202). The emergence in some cultures of early and recurrent swarms of such great numbers of these cells would make it seem improbable that they are entirely the progeny of those relatively few histiocytes carried over with the explant in the peri- and endomysium. Most of them, however, emerge from the explant interiors. Serial sections of supravitally stained explants resemble, in the presence of similar cell forms, sections of the border regions of infarcted muscle. In addition to necrotic and proliferating muscle fibers and "fibroblasts" there can be distinguished numerous plasmodial sarcoblastic slips and some unattached fusiform cells or straps with the same cytological characteristics, small multinucleated "buds," some larger racquet-shaped or clubbed symplasts (giant cells), numerous large, rounded macrophagic cells. Most of the latter have accumulated trypan blue granules, but a few have not. Dye is almost never found in the sarcoblasts and straps. Muscle explants, taken for sectioning at successive intervals, during the first few days show evidences of mitotic activity and mobilization of those "fixed" histiocytes normally resident in the perimysial connective tissue. But the formation of some trypan-blue storing cells from the surviving nucleated parts of degenerated fibers with which they still remain in continuity is also suggested by such sections. These elements possess typical rosettes of dye-granules, and remain part of the necrotic fiber, within its sarcolemma (Fig. 206).

The actual birth-process of large macrophage-like corpuscles can be occasionally followed from its beginnings in certain sarcoblasts of the outgrowth zone of the living cultures, where direct visualization is possible. Refractile granular sarcoplasm may be seen to gather about a nucleus in a sarcoblast fiber making a focal node either at the tip, where this node may break off, or along the course of the fiber, from which it either buds out, trailing a gradually attenuating filamentous attachment or protoplasm, or simply splits out of the sarcoblast ribbon, thus fracturing it (Figs. 203 and 204). This budding-off of free motile cells from sarcoblasts is an infrequent occurrence. But under certain unusual conditions large numbers of isolated cells may be found to have formed at the seeming expense of the sarcoblasts. It therefore appears likely that at least some of the large round cells can be of muscle origin and, as we have seen, most of these become structurally and behaviorally indistinguishable from macrophages.

From the evidence at hand it is reasonable to conclude that the large, round, motile free cells which occur in injured muscle *in vivo* as well as in tissue culture may originate both from the histiocytes ("resting wandering cells") of the connective tissue and, to some extent, from surviving elements of the muscle fiber proper. Having undergone this transformation, they cannot be told apart.

These cells from mature mammalian muscle are in most respects similar to those which Chèvremont [20] has described as originating from chick embryo muscle in culture, and which he has designated "histiocytic." Pogogeff and Murray [89] noted the occurrence of large "macrophage-like forms" in rat muscle cultures, but could not be certain of their fate.

The development of macrophagic cells from muscle fibers is not a unique instance of the "histiocytic transformation" of cell types. The transition of blood monocytes to macrophages, epithelioid cells, and giant cells *in vitro* has been known since 1925 [18, 69] and has been studied histochemically by Weiss and Fawcett [113]. Lymphocytes [76, 110], fibroblasts [36, 81, 103], embryonic mesenchyme [34], vitelline membrane cells [104], smooth muscle, iris [20, 21], and Schwann cells [117] are all alleged to be capable of becoming macrophagic cells in vitro. The transformation of fibroblasts or of other cells to true macrophages has been denied by some [82] on the basis of morphological observations in fixed tissue culture preparations after supravital staining, but without the advantage of sequential observations of living cells.

SOME PROPERTIES OF THE MACROPHAGIC CELLS. In morphology and behavior the large, actively motile cells from muscle appear almost identical with those derived from blood buffy coat, with those found in chick fibroblast cultures, and with those described from other origins. Most of them store supravital dyes like trypan blue in a rosette which circumscribes a large, poorly stained, relatively nongranular, juxtanuclear area (cell center; Golgi region) (Fig. 207). This inner central zone is occupied by a diastase- and pyridine-resistant material stained by the periodic acid-Schiff procedure (Fig. 208). This same locus is also the site of strong acid phosphatase activity with both Gomori and azo dye tests (Fig. 209). Most of the histiocytoid cells have a striking granular refractility. *In vivo,* early observers [110] noted the tendency of these "muscle corpuscles" to become fatty, and indeed, the relationship of cells of the histiocyte family to lipide metabolism suggested that identification and localization of their intracellular lipide would be profitable. Both the round cells of the tubes in the rabbit-lesions and the similar histiocytoid cells of the muscle cultures contain abundant, diffusely distributed material stainable with sudan-black B and variable numbers of intensely sudanophilic globules (Fig. 210). The dispersed material contains plasmal and phospholipide; most of the small droplets and granules contain neutral fat having unsaturated fatty acid, and in cultured cells the droplets tend to be distributed in an arc about the cell center.

We have found these features equally characteristic of macrophages in lesions of rabbit muscle, in tissue cultures of rat muscle, and also in macrophages of chick fibroblast cultures. All of these are histochemically identical with the macrophages derived from chicken blood buffy coat as reported by Weiss and Fawcett [113]. On the basis of their cytologic characteristics and their contents of certain lipides, Chèvremont [20] concluded that the macrophagic cells arising in cultures of a variety of embryonic tissues could not be distinguished by methods then at his disposal, and that all histiocytic-macrophagic cells from whatever source were essentially identical. Weiss [114], however, believed that the histiocytic macrophagic cells retain signs of their descendancy, so that a macrophagic cell derived from Schwann cells, for example, can be ultimately distinguished from a macrophagic cell derived from endoneurium or blood monocytes. The critical test presumably would be one evoking their "prospective potencies."

Our experience confirms the impression that the macrophagic cells of whatever origin are fundamentally similar not only in morphology and apparent activity, but also chemically. It is important to note that the parent cells in each case, i.e. the cells which are transformed (monocytes, resting histiocytes, sarcoblasts or muscle fibers, chick fibroblasts) are devoid of the characteristic polysaccharide and cytoplasmic acid phosphatase of the active macrophagic cells which are supposed to arise from them, and have different lipide contents and distributions. It is evident that the histiocytic transformation involves considerable change in composition, and not merely changes in the disposition of pre-existing components. Whether these changes may be taken simply as the consequences of a redistribution of a molecular population present in both parent and transformed cell, as Weiss would hypothesize [116], can hardly be proved at a microscopic level. The retention of the basic genetic equipment of the parent cell by such histiocytoid cells deriving from a variety of tissue types, in spite of the cytological and histochemical identity of the histiocytoid cells, would be shown only by proof of the reversion of the latter to the parent form.

FATE OF THE MACROPHAGIC CELLS. The macrophagic transformation has been thought of as a true modulation and therefore reversible [114, 116], although this reversibility has rarely been demonstrated. The macrophage-histiocyte-fibroblast states have been supposed to be interchangeable [11, 27], and according to Maximow [76] and to Bloom [11], the so-called polyblasts derived from blood mononuclear cells may become reticulin-producing fibroblasts. It should be repeated that in assessing the nature of a transformed cell it is not sufficient to follow vicissitudes of form, but functional and cytochemical criteria should also be applied. Macrophages may undergo remarkable changes in form [18, 31, 113] into spindle-shaped cells hardly distinguishable visually from fibroblasts; identification can be made if histochemical properties, such as those mentioned, or functional attributes, such as mode of vital dye segregation, or reticulin or mucopolysaccharide [48] production, are also invoked as criteria.

In the ischaemic lesions of rabbit muscle most of the macrophagic cells, gorged with debris, wander out of the cell tubes and make their way through the interstitium to the efferent lymphatic channels. In the tissue cultures, most develop watery vacuoles and appear to disintegrate. In the healing lesions (10th–16th day) of rabbits, and in culture, these histiocytoid cells tend to accumulate lipide, and in sections groups of them may somewhat resemble small lobules of embryonal adipose tissue. However, in the experimental material investigated, most of them seem to disappear from the scene. At present the possible potencies of the large macrophage-like cells from muscle is unknown, and whether they can revert to muscle, or become fibroblasts or fat cells, or are invariably at the end of their careers still remains to be discovered.

CAUSATIVE FACTORS IN THE TRANSFORMATION TO MACROPHAGE-LIKE CELLS. To Chèvremont, the histiocyte represents a special and more resistant physiological state which many types of differentiated cells can assume as an adaptation to changed external conditions [21]. Some indications of the conditions bringing about the transformation into macrophagic cells have been obtained in vitro. The addition of choline and related quaternary bases to tissue cultures was reported by Thomas [105] and subsequently Chèvremont [22] to enhance this transformation. Chèvremont and his associates have regarded choline and similar bases as the determining substances; Törö [107] has designated histamine as a transforming agent. Such substances may act synergistically with local factors. An obvious topographic feature of the "muscle-cell" tubes of the infarcted muscle bears on this point. The macrophagic round cells are concentrated in the tube interiors, close to the necrotic zone where blood supply and gas exchange are poorest, whereas the long sarcoblastic sprouts are found closer to the vascularized zone, and at the tube peripheries, where nutrition and oxygenation are presumably better. It seems possible that not only chemotactic substances related to muscle necrosis, but environmental factors like those mentioned may determine which cell types prevail in any locus *in vivo,* although Chèvremont [20] has denied that pH, oxygen tension, or the presence of plasma influence the production of macrophagic cells in tissue culture.

OTHER FORMS OF ISOLATED FREE-CELLS OF MUSCULAR ORIGIN, MODULATION AND METAPLASIA IN MUSCLE. A few of these large free cells have less granular, often amphophilic cytoplasm, larger nuclei and nucleoli, and usually poorly developed cell centers. They may have variable, small or indetectible acid phosphatase activity and polysaccharide substance. They may take up small amounts of supravital trypan blue, or remain entirely unstained. These cells appear to be transitional forms to the fully developed macrophage. Some may be thought of as cells originating from muscle, which have not acquired all of the properties of the macrophage. The accumulation of acid phosphatase, for example, seems to be associated with activation of cells to the phagocytic macrophage type [30, 113]. Muscle nuclei and sarcoplasm may also give rise to giant cells. Many appearances in histologic sections, as pictured by Dawson [26], suggest the formation of giant cells directly from the surviving peripheral nuclei and sarcoplasm of degenerating fiber fragments cut off by fibrous tissue.

The other apparently isolated cells of the tubes in the healing muscle infarct are fusiform, nonphagocytic elements usually with basophilic cytoplasm. They are generally found at the periphery applied to the inner wall of the tubes. Many of them are linked by slender cytoplasmic connections into sarcoblastic ribbons or short straps as Le Gros Clark [63] reported, but some appear to be individualized sarcoblasts. They are comparable with the plump, spindly elements with "stiff" highly refractile cytoplasm into which sarcoblastic ribbons of the outgrowth zone of tissue cultures break up, in such circumstances as starvation or exposure to elevated temperature. De Renyi and Hogue [28] elicited the same phenomenon by mechanical stimulation of sarcoblast fibers, and have referred to the new cells as "myoblasts." Mauer [75] also found from his study of sections, that muscle fibers of the explants in tissue culture "dedifferentiated" into single spindle cells. Their fate is also in some doubt; most authors [2, 98, 109] believe that such single elements subsequently link up to form fibers, and are therefore myoblasts, while others deny that this occurs [8, 63]. In some foci such spindle cells occasionally fan out into zones of adjacent fibroplasia, in which it may be impossible to distinguish them morphologically from fibroblasts. This is particularly the case in areas of severest ischaemia, where fibrosis is prominent. Indeed, Betz [8] insisted that much of the fibrosis consequent on the profound ischaemia resulting from total devascularization of a muscle was due to the formation of such spindle cells from surviving muscle elements, and their conversion to fibroblasts. This author considered them to be elements of sarcous origin capable only of becoming histiocytic cells or fibroblasts. Mauer [75] also held that the single fusiform cells of muscular origin become connective tissue cells, i.e. fibroblasts, in culture, a process he referred to as "regressive metaplasia." Some authors in the older literature, and more recently Tower [108] and Altschul [3] have held that the islands of adipose tissue and the diffuse fibroplasia which are a constant finding in neurogenic muscular atrophies are derived from an actual metaplasia of muscle cells. This is denied by Adams, *et al.* [2].

The name which we apply to these transformations depends upon our ability to differentiate between cells of similar appearance in biologically meaningful terms. In the absence of such information the possible metaplasia of muscle cells, i.e., their transformation to other fully differentiated types, remains doubtful.

4. *Regeneration: the Sarcoblasts*

Useful regeneration is effected chiefly by wide sarcoblastic ribbons which bud forth from the stumps or ends of the still intact fibers of the collaterally vascularized muscle. The terminal segments of the surviving fibers lose their cross striations, and in preparation for the ensuing growth there occurs intensive enlargement and proliferation of the nuclei which may congregate in groups at the fiber end, dispersed between many myofibrils, or align themselves in queues at the periphery under the sarcolemma. Basophilic cytoplasm gathers about the nuclei, and the emergent sprouts grow out either as continuous, distally tapered, plasmodial bands whose proximal parts are similar in diameter to the parent fiber (terminal outgrowth), or along the periphery as narrower ribbons which cleave to the inner side of the sarcolemmal endomysial tubes (peripheral outgrowth).

But sarcoblastic slips also take origin from the surviving nuclei and sarcoplasm of partly degenerated segments whose contractile parts have succumbed. Such degenerated fibers, as we have seen, in slightly different conditions can also give rise to free isolated cells and to muscle giant cell or multinucleated "bud" forms. The sarcoblasts arising as a result of this "dissociative degeneration" are always slender ribbons or straps which lie against the inner aspects of the tubes.

GROWTH. Sarcoblasts of either origin, growing as long ribbon-like bands, flow upon the structural guide lines provided by the endomysial planes of the tubes. In its dependence upon an organized stromal scaffolding which reproduces the tissue pattern, successful regeneration of skeletal muscle is analogous to that of other tissues, such as liver and peripheral nerve, for example, and it has an intrinsic growth capacity not much inferior to these. When the sarcoblasts meet with an obstruction, or when segments are cut off by fibrous tissue, they tend to form syncytial masses or buds, the muscle giant cells. Otherwise, they grow as continuous tapered strips with somewhat expanded ends possessing distal lanceolate extensions. Although sarcoblasts are not usually recognizable until the third day, in some specimens the ribbons have already attained lengths exceeding 4 mm. by the fourth day after operation. Thereafter lengthwise growth occurs more steadily at an average rate of 1.2 to 1.7 mm. per day up to the 14th day, according to Le Gros Clark and Wajda [65]. The growth in diameter of sarcoblasts is more difficult to assess, owing to the presence of both wide and slender sarcoblasts, the differences in size at different levels of the tapered fibers, and the very considerable differences in diameter of the regenerating fibers. Nevertheless, our measurements of similarly differentiated fibers roughly follow a two phase pattern and show a fairly steep increase from an average range of 10–20 μ during the 6th to 14th days after injury, and a slower more gradually sustained rise thereafter over a period of 10 weeks or more, when the average diameters of new fibers are 15–30 μ. There is a correlation between fiber diameter and its differentiation, for these increases seem to occur only with cross striation. The slower late increase reflects the increase in the contractile apparatus.

An early burst of very rapid translatory growth, followed by a slower and much more gradual increase in length of sarcoblasts, is also observed in tissue cultures of muscle.

NUCLEAR DIVISION. Preceding and during the rapid growth phases, nuclear multiplication is correspondingly great; yet mitotic figures have not been found in plasmodial sarcoblasts at this stage, either *in vitro* or *in vivo,* even after colchicinization [4, 44, 63, 102]. Most observers [2, 8, 20, 63, 79, 80, 88, 89, 111] have similarly failed to discover mitotic figures in growing muscle. Many appearances indicate that nuclear multiplication in the sarcoblast occurs entirely by amitosis. Contiguous nuclei with apposed membranes lie in pairs, triplets, and quadruplets or large clusters which seem to have resulted from fission of an original nucleus (Figs. 217–219). The nuclear membranes are folded into sharp incisures or clefts which extend into the nucleus as partitions or constrictions apparently separating adjacent nuclei, or parts of nuclei, and suggesting that the amitotic nuclear division is brought about in this way (Fig. 220).

Study of regenerating sarcoblasts leads to the conclusion that the amitotic nuclear division is the unique and regular manner of repartition of the nuclear contents and consequent nuclear multiplication of the developing sarcoblast. The process seems to possess physiological validity, and the fact that it "may seem to infringe upon the current theories of cellular heredity is certainly no sound reason either for ignoring its existence or for relegating it to the very convenient category of a degeneration phenomenon" [86]. Actually, nuclei of mature muscle, on the basis of biochemical analysis and nuclear counts [13, 55], are believed to possess the somatic or diploid quantity of desoxyribonucleic acid. Preliminary microphotometric measurements of the Feulgen-DNA content of individual sarcoblast nuclei made in our laboratory, indicate that they are mainly of the 2C or diploid class, coinciding with the same predominant class among the free cells. The manner in which this precise distribution of hereditary material to daughter nuclei is effected by the seemingly inexact process of direct division remains to be disclosed by a sequential study of the mechanism of amitosis in living sarcoblast nuclei, coupled with cytophotometric evaluation of their DNA contents.

Amitosis as a usual mode of nuclear multiplication is probably related to the existence of the plasmodial or symplastic state, in which cytoplasmic cleavage does not follow nuclear divisions.

5. *Redifferentiation*

The events of differentiation can be followed most clearly from the earliest phases in the slender sarcoblastic slips, whose development will be described. At least four major optically visible developments in the structural cytodifferentiation of the sarcoblast can be distinguished: the prefibril, fibril, cross-striate, and maturation stages. These distinctions are made because of the appearance of specialized structures, such as fibrils or discs, or other morphological changes which serve as markers of the progress of differentiation, but which might also be regarded as the products of antecedent chemical events which Huxley called chemodifferentiation [59]. It is no longer necessary to admit any sharp distinction between structure and constitution; rather, the task in the study of cell differentiation is to make the correlation between emergent structure and changing composition by means of the precise localization of substances.

PREFIBRIL STAGE. The early plasmodia are slender, richly nucleated slips with homogeneous basophilic cytoplasm (Figs. 211, 212). Even at this stage the sarcoblasts of ribbon form may show well-defined birefringence of positive sign. The nuclei are disposed into axial rows, usually lying in contiguous focal heaps. Each has two to three times the estimated volume of a resting muscle nucleus. These nuclei are strikingly vesicular, and have 2 to 4 nucleoli (plasmosomes), one of which is typically enlarged and elongate. The nuclear membranes and their infoldings are firmly outlined, deeply stained with basic dyes and with the Feulgen reaction. The cytoplasm possesses long filamentous mitochondria, regimented in parallel; this and the birefringence bespeak an oriented submicroscopic structure (Fig. 221). The prefibril sarcoblasts show good tetrazolium salt reduction in the test for the succinic dehydrogenase system, as might be expected from their mitochondrial content, sharply differentiating the regenerative from the nonviable areas. Their cytoplasm contains much diffuse plasmal, but little other stainable lipide. Histochemical tests show the entire cytoplasm to be very rich in pentosenucleic acid (PNA), as are the nucleoli (Fig. 211). Biochemical analyses of whole anterior tibial muscle at graded intervals after ligation reveal a steep rise in the total PNA concentration to about double the control values, beginning at about the 4th day [56]. This is just when the first prefibril sarcoblasts have begun to grow out in ribbons, after the subsidence of the initial inflammatory reaction. This elevated gross concentration of PNA is maintained throughout the next phases of differentiation and maturation. It is of interest in this connection that the activity of nucleoside phosphotransferase, an enzyme which catalyzes the synthesis of nucleotides by transfer of phosphate from organic phosphates to nucleotides, undergoes a fivefold increase per gram of muscle in regeneration (7th day after ligation) as compared with normal control muscle [14]. Braverman and Chargaff [14] have observed that this enzyme is significantly increased in a number of growing systems engaged in synthesizing nucleic acid and protein. Nonspecific acid phosphatase is not increased to any extent in regenerating muscle [14], nor is alkaline phosphatase histochemically demonstrable in the sarcoblasts at any stage.

FIBRIL STAGE. The next morphological advance in the differentiation of the slender sarcoblasts is the appearance of primitive myofibrils: continuous homogeneous lines in a denser ecto- or sarcoplasm on either side of the axial line of nuclei (Figs. 213, 214). The nuclei now lie in a central column of somewhat foamy or reticulated cytoplasm in which glycogen can usually by localized. PNA is now apparently more concentrated in the perinuclear central axial cytoplasm. By the late fibril stage the nuclei have attained their greatest numbers and most extensive development, with average volumes nearly 1.5 times as great as those of the enlarged, activated nuclei of the early proliferative and prefibril stages. They are usually more sacculate and burgeoned as a result of more numerous and deeper membrane infoldings. The larger rodlike or baton-shaped nucleoli have become more massive, and almost always can be found to be in contact with the nuclear membrane (Fig. 212). They are presumably the bodies in the nuclei of regenerating rabbit muscle referred to by Millar [80] as "rhombosomes." The behavior of this nucleolus suggests an important function probably related to the synthetic events in the cytoplasm, of which the primitive myofibrils are visible evidences. The latter are uniformly birefringent, rather dense filaments which occur in the cytoplasm in pieces of varying length along the course of the developing ribbons. Such primary fibrils have been described in the differentiation of embryonic myoblasts [33, 43, 111]. It is of historical interest that many of the classical cytologists [32, 68, 84] believed that these myofibrils were derived from the actual fusion of mitochondria or granules. The prefibril sarcoblasts, in their ribbonlike continuity and weak birefringence, give evidence of an oriented submicroscopic structure even before fibrils are seen. The longitudinal array of their mitochondria (one of the features which led earlier observers into assuming their subsequent transformation to fibrils) is the result, as Pollister [90] deduced, of these organelles being passively regimented into file by a fibrillar organization of the sarcoplasm taking place below the limits of resolution of the light microscope. The visible myofibrils pre-exist as protofibrils which become optically manifest only after they have grown to resolvable dimensions. All the new myofibrils in regenerating mammalian muscle seem to arise this way from the sarcoplasm, rather than, as in the trout, by splitting of the primary myofibrils [52]. This concept of the organization of protofibrils, astutely put forward in 1911 by Heidenhain [51], is amply supported by electron micrographs of amphibian myoblasts in which, according to Porter [91], protofibrils "polymerize out" of the sarcoplasmic matrix. More details on their formation, in particular on their relationship to the "growth bodies" [91] and the sarcosomes [7] and their aggregation to primitive myofibrils, would be desirable. Although the mitochondria or sarcosomes apparently do not participate in the formation of protofibrils, they nevertheless enter into such close structural relation to the myofibrils that some believe these components to exist in a symplastic state [112]. Fibrillation may be taken as a morphological indication of the formation of contractile proteins. It is well under way as early as the 5th day after ligation in the regenerating rabbit muscle. The inability of Csapo and Hermann [25] to extract contractile protein from chick embryo somites before the 9th day, although fibrillation and striation [111] and contractility are established by the 7th day of incubation, suggests that morphology may be a more sensitive indicator of the first occurrence of these proteins in the early phases of development.

CROSS STRIATION. The next step in redifferentiation is marked by the development of periodic basophilic thickenings along the heretofore homogeneous myofibrils, while at the same time the periods between the thickenings become less evident. The thickenings are strongly birefringent, and are evidently the first A (or Q) discs; the alternate intervals are the primary I discs (Fig. 215). The H (M) band appears to be formed very close upon the deposition of the A, which it separates into two halves. The A discs may be laid down upon the undifferentiated myofibril very soon after it appears and before other myofibrils have formed in the sarcoplasm, but more often cross striation is not seen until a few myofibrils are present (Fig. 216). Not all of the myofibrils in any sarcoblast attain these new structures at once; nor in any single myofibril is its entire length necessarily differentiated at the same level. Cross-striated and plain myofibrils may exist side by side. Cross striations first appear in some regenerating slips on the 6th day after infarction and increase markedly thereafter. Following this alternate striation, sometimes quite late, is the bisection of the I disc by the Z line. Usually, in such young fibers, a sarcolemmal membrane can be made out with the PAS stain. Fibrillation and cross striation thus show themselves to be stepwise processes. This is also the case in embryogenesis [43, 111]. Invoking the evidence put forward by Hanson and Huxley [50], it would appear that actin is first organized structurally as the continuous fibril and that myosin is subsequently localized to the A band, which according to these authors, is its proper site in the mature fiber. This organization of these substances into structures may not, of course, parallel their syntheses. Indeed, the analyses of Csapo and Hermann [25] of chick embryo muscle indicate that myosin formation either precedes that of actin by four days or else the actin is not at first combined as actomyosin.

While cross striation is taking place, new myofibrils are constantly being laid down, and the sarcoblasts are growing steadily in length and diameter. The hypertrophic nucleoli, at this stage, show a tendency to elongate and become acicular (Fig. 219). In the sarcoplasm, PNA can be demonstrated with basic dyes, and its concentration (PNA/DNA ratio) as shown biochemically remains at nearly the same high level throughout the time when cross striations are forming in most sarcoblasts. The basophilia of the new A discs and the presence of phospholipide in the primitive I discs are demonstrable in the striations of the sarcoblast as soon as formed, as they are in the adult fiber [29].

MATURATION. The events occurring in the transformation of the cross-striated sarcoblast to a mature muscle fiber comprise this process. Maturation begins after the first contractile equipment has been fashioned in those sarcoblasts destined to prevail, and becomes the principal phase of differentiation in the regenerating muscle from the tenth to fourteenth day forward. During this phase the sarcoblast gradually fills with striated myofibrils and increases chiefly in diameter, but the volume of sarcoplasm is not much increased. The nuclei, having stopped dividing, have been separated by the differential augmentation of fiber size, and now tend to be displaced peripherally by the increasing contractile mass of fibrils. They recede in volume and lose their membrane folds; their nucleoli are reduced to spherules, although still larger than in adult resting nuclei; their basichromatin becomes somewhat more prominent, and the diminished nuclear sap is now slightly basophilic. The I discs and sarcoplasm become increasingly acidophilic. In three to four weeks after infarction the regenerated area consists of sinuous new muscle fibers of small and quite variable diameters (from ¼ to ¾ those of mature fibers) and with increased numbers of nuclei, many of which have not attained the periphery (Fig. 223). In the regenerated infarct, in which there is some fibrosis and presumably disturbed reinnervation, the new fibers do not mature completely or grow to the size of comparable normal muscle fibers. In lesions of other kinds, such as regeneration following Zenker's degeneration of Coxsackie virus infection, in which the sarcolemma is preserved, complete maturation and restitution of normal fibers is the rule.

The maturation phase is characterized by large increases not only of contractile proteins, as evidenced by the increase of striated fibrils, but presumably of some sarcoplasmic proteins as well, including the basic myohemoglobin, as indicated by increased affinity for acid dyes at somewhat higher pH. PNA becomes more difficult to detect by means of basic dyes, probably owing to its dilution or dispersion in the enlarging fiber (i.e. decreased concentration), to competitive inhibition of its dye binding sites by protein, or both.

In the development of chick skeletal muscle there is a sudden sharp rise in actomyosin concentration and intracellular protein at about the 12th day of incubation. The bulk of the actomyosin, however, is synthesized only after about the 18th day [25]. There is no evidence that these contractile proteins are qualitatively different in earlier and later stages [25]. It has been proposed by Hermann that the mechanisms involved in the formation of the first traces of these proteins may be different from those concerned in their large-scale elaboration at a later stage of development. The rapid increase of the energy-rich phosphate esters, phosphocreatin and ATP in the chick, is also relatively late (14th–16th day), occurring after the first marked augmentation of contractile protein [54]. The PNA:DNA ratios, however, remain within a narrow range after the 11th day [53]. The bulk production of the specific structural and functional contractile components of muscle thus does not take place until a relatively late stage of ontogeny [25, 53]. This "terminal phase" of quantity production of specific cytoplasmic structures, which is accompanied by increase in cell mass [53], corresponds to our stage of maturation in regenerative redifferentiation, with its gradual increase of fiber diameter and accumulation of myofibrils.

NUCLEAR DIFFERENTIATION. Of special importance are the changes of nuclear size and structure which occur in the differentiating sarcoblast, and which are related to the phases of cytoplasmic activity. The occurrence of nuclear changes in developing myoblasts as remarkable as those taking place in the cytoplasm was commented upon in 1904 by Eycleshymer [33], who concluded that the nuclear material must play an important part in cytoplasmic syntheses. This, indeed, is a currently accepted concept toward which several lines of evidence contribute. The marked increase of nuclear volume, and nuclear vesiculation, accompanied by dispersal of the basophilic chromatin are among the earliest changes preparatory to sarcoblast growth. They occur before the advent of maximal cytoplasmic basophilia. These almost certainly are reflections of large increases of nuclear nonhistone protein. Cytoplasmic differentiation of the sarcoblast is preceded by additional swelling, new changes in the nuclear membranes, and nucleolar hypertrophy (Fig. 219). Nucleolar size and nuclear membrane infolding are greatest during fibrillation. Although no further increase in volume occurs and some regression of nucleolar size begins in the early cross-striated elements, nuclear maturation occurs only slowly thereafter and return to the size and structure of the adult nucleus probably does not take place until after cytoplasmic maturation has been completed and the fiber has attained normal size. Nuclear function, as judged by nucleolar size, vesiculation, and membrane chromaticity seems to be important also during the slower deposition of cytoplasmic structures occurring in the maturation phase.

GROWTH AND CYTODIFFERENTIATION. It is a widely held assumption, and one indeed for which there is much presumptive evidence, that cell proliferation and differentiation are incompatible [101], that cell proliferation inhibits the building of structures peculiar to their type [38], and that reproductive quiescence is a necessary condition for differentiation, which must take place between or after divisions [37]. The universal applicability of this rule has been doubted [116], and if, instead of division, protoplasmic increase were used as a measure, an inverse relationship with differentiation would indeed be hard to show in many cases.

Bursts of proliferative activity alternating with periods of differentiation are supposed to occur in embryonic development. Spurts of proliferation apparently occur in the embryonic development of muscle [53]. But the extent to which mere abeyance of division is responsible for differentiation, or indeed the nature of the relationship between proliferation and differentiation, remains problematical.

In the regenerating muscle the most rapid growth occurs in a spurt during the period of least apparent differentiation, i.e. in the prefibril sarcoblast. Nevertheless even at this stage of active nuclear reduplication and cytoplasmic increase the submicroscopic groundwork for the subsequently visible steps of differentiation are being laid. The early phases of fibrillation and striation also occur during a time of diminished, but still active growth. The production of the larger quantities of specific cytoplasmic protein in the maturation stage, however, occurs after nuclear replication has ceased, and is accompanied by a slow, gradual increase in the rate of augmentation of fiber size.

The general rule of the inverse relationship between growth and differentiation is not absolute. The first steps in sarcoblast redifferentiation, as we saw, proceed during an active growth phase, although, of course, it cannot be excluded that the specific cytoplasmic structures are actually laid down in the intervals between short successive spurts which together might constitute the phase of rapid growth. Preliminary experiments on the growth of fibroblasts *in vitro* [48] suggest that the specific function of acid mucopolysaccharide production by these cells is positively related to their growth. It may be that growth, and the differentiation of special structures and functions, are in some ways complementary, as well as mutually competitive, although not mutually exclusive. The development of cells, as Rusch [94] states, "is a resultant of the outcome of the dynamic balance between the functions of cell reduplication and of special function." In other terms it might represent the effects of the relative distribution of nutrients to the one or the other activity at any time [94, 116]. Earlier steps in differentiation are quite compatible with rapid proliferation, both processes being sustained simultaneously, but the absence of nuclear reduplication (autosynthesis) in the terminal stages, associated with large scale production of specific cytoplasmic products, may represent the diversion of substrates entirely to specialized cytoplasmic equipment (heterosynthesis). Access of such materials to the cell must affect the course of differentiation. For example, if the sarcoblastic ribbons, even in older specimens, are followed over considerable length from the intact side toward the persistent necrotic or fibrotic foci, a diminishing gradient of both size and differentiation is seen near the ischaemic parts. Even in the same fiber, there may be varying phases of development seemingly influenced by such local environmental factors as oxygenation, nutrition, and tissue products.

The part played by some specific extrinsic factors in regulating redifferentiation, and the contribution of autogenous substances present in the intact organisms can to some extent be evaluated in tissue culture.

THE DIFFERENTIATION OF SARCOBLASTS IN VITRO. Sarcoblastic ribbons of mammalian [89] and chick embryo origin [15, 41, 70] in tissue culture are capable of forceful spontaneous contraction in the absence of any optically visible cytoplasmic fibrils or striation.

Nevertheless, their continuous ribbon form, their weak positive uniform birefringence and gelated sarcoplasm, and their response to colchicine by disruption and loss of consistency [44] betray a submicroscopic fibrillar structure. Pogogeff and Murray [89] showed that the mammalian sarcoblasts develop longitudinal and then cross striations, the latter usually alternate light and dark bands, after about 20 days *in vitro.* We have found that with continued sojourn *in vitro* under conditions favoring differentiation, many sarcoblasts proceed to develop a complete set of cross striations: Q discs with H lines and I discs and finally Z lines. A curious feature of these cross striations is their abruptly localized distribution through a limited sector or part of a sarcoblast. They may appear, disappear and recur [89], or be lost in one sector and develop in an adjacent locus, behavior which can be described as modulation of the sarcoblast, or as reversible phases of partial "dedifferentiation" and redifferentiation. Such cultures have been maintained *in vitro,* some after multiple subcultures [89], for many months. It is evident therefore that skeletal muscle is capable of regenerative growth, structural differentiation and contractility independent of any innervation, and in the absence of any homologous humoral factor. These phases of cytoplasmic differentiation in the sarcoblast are therefore independent of organismal control. If it could be shown that they also take place in the absence of other cell types *in vitro,* the sarcoblast would be referred to as self-differentiating. Only the final stage of maturation, with its bulk production of contractile materials, does not occur *in vitro.* It is probable that connection with the central nervous system, or other trophic factors supplied *in vivo,* or both, are necessary for this development.

ASPECTS OF SOME CURRENT CONCEPTS OF CYTODIFFERENTIATION. Even this very incomplete sketch of sarcoblastic redifferentiation serves to exemplify that differentiation is complex, and progresses in stepwise or seriate fashion. It requires at the outset the formation of a specialized protoplasm in the mesodermal cell which is in turn capable of manufacturing and ordering the basic chemical equipment (myboblast or prefibril sarcoblast) which can elaborate specialized structures (such as protofibrils and myofibrils of the fibrillated sarcoblast), whose formation must precede the orderly deposition of still other structural substances (cross striation) until all the equipment of the terminally differentiated cell (muscle fiber) is present and arranged in proper relationship. Once the process has begun it can probably follow only one direction at any one time ("exclusivity" in Weiss' definition [116]), finally to result in a terminally differentiated cell which is in a class sharply distinct from other differentiated cells ("discreteness").

In chemical or molecular terms the events of differentiation have been represented as a concatenated series of mutually dependent, successively triggered metabolic reactions, involving in series the sequestration, segregation, inhibition, or elimination of some of the originally present systems of the undifferentiated cell and the concomitant activation of others. Such hypotheses have been offered by several authors [91, 92, 93, 116] and have as their most important tenet the dependent, sequential, and orderly nature of interlocking reactions. Activation of some metabolic systems by others might be accomplished by the accumulation, through the activity of the prior system, of certain substrates to critical levels which may thereby induce the formation of new enzyme systems ("induced enzyme formation" [91]). Rose [93] has stressed the successive inhibition of originally present, gene-initiated reactions by the collection of inhibitory metabolites, as the principal mechanism of differentiation. Even more important may be the segregation or selective grouping of certain key compounds, presumably at interfaces, thereby assuming a controlling influence on the further reaction pattern of the cell by governing the influx and disposition and availability of other molecules [115, 116]. According to this theory of "molecular ecology" proposed by Weiss, the primary steps toward differentiation involve changes in localization of certain molecular "species"; compositional changes such as competitive depletion or concentration of other "species" would necessarily ensue. This and similar theories have the merit of focusing attention on the predominant importance of the localization and segregation of reacting systems for the development of ordering conditions which then permit a sequence of dependent reactions to occur. Such spatial segregation is an indispensable condition for the temporal evolution of a patterned process. This would be inconceivable in a random state, such as a homogenate. These theories at present remain abstractions, which to become fully applicable to any case, must be invested not only with specific chemical meaning, but must also be defined in terms of ultrastructure.

In cognizance of the indispensability of some cytoplasmic segregation mechanism for differentiation, it has sometimes been postulated that this has a definite particulate basis [99]. Moreover, since various types of cell lines tend to maintain their properties through subsequent growth and division [119], and in view of the obvious precision of segregation mechanisms, some authors have justifiably hypothesized that the persistence of cell lineage is due to the self-reproduction of such cytoplasmic particulates [99]. Such specific particulates might be hereditary, or transmitted during cellular interactions. Mitochondria, microsomes, and "plasmagenes" [120] have at various times been assigned these roles but without conclusive evidence, so far as the mammalian cell is concerned.

If it can be assumed that in any organism cells which undergo divergent differentiation have the same genetic endowment ("genetic equivalence" [119]), it follows that the changes of differentiation must involve the extragenic components of the nucleus, as well as the cytoplasm. Within the nucleus, the histones have sometimes been assigned the role of differentiating substances, supposedly by regulating genic "expression" [100]. Recent studies have also disclosed differences between the nuclei of actively dividing, and of resting, presumably differentiated, functioning cell populations with respect to the nonhistone protein content of their nuclei in interphase [9]. The function of the nucleus in originating and perpetuating differentiation is still unclear. It is obvious that both the structure and composition [9] of the nuclei change in the course of differential development in any cell line, as illustrated in the case of muscle, and it has most often been assumed that these changes are a reflection of differences in their cytoplasmic environments. In the light of current cytochemical knowledge, the assumption that nucleus and cytoplasm interact to bring about the changes recognizable as differential seems to be a more fruitful concept.

Factors originating extracellularly, and the direct interactions of different cell types which influence the course of differentiation, are well known to experimental embryology and documented in an abundant literature. For instance, fragments of nonviable cartilage have been reported to effect the differentiation of fibroblasts of other origin to chondroblasts *in vitro* [37]. Similar inductive effects have been reported *in vivo* [99]. Examples of direct inductive cellular interactions influencing differentiation in embryonic life are very numerous. Inductive interactions of mammalian cells in culture have been demonstrated by Grobstein [47], who has found that epithelial morphogenesis (tubule formation) depends upon specific properties of the mesenchyme cells with which they are put into association. In these interactions the cells which induce differentiating changes in other cells do not themselves undergo profound alteration [99]. Of particular interest is the report by Rose, that the presence of adult tissues inhibits the differentiation of the corresponding embryonic tissues [93]. Physical and mechanical forces also influence tissue differentiation, especially in the connective tissues [62]. Most such agencies have been presumed to exert their influence primarily on the cytoplasm of the differentiating cell [115], but both the locus and mechanism of their action on an intracellular level require elucidation.

Among the mechanisms proposed for the cellular interactions or inductions which give rise to dependent differentiation are (1) the liberation of diffusible substances, which may be unstable and short lived; (2) the transmission of cytoplasmic particulates, which may be self-replicating; and (3) cell-to-cell contact, in which the formation or rearrangement of molecules would resemble antibody production occurring in response to an antigen.

THE PLASTICITY OF MUSCLE PROTOPLASM. We have seen how, after an injury which destroys the more sensitive contractile parts of the fiber, the viable nuclei and sarcoplasm proliferate and can be transformed, depending upon environmental conditions, into (1) free cells, which may become macrophage-like, and spindle cells, some of which are single sarcoblasts and others of undetermined potencies; and (2) sarcoblast fibers and muscle buds or muscle giant-cells. The protoplasm in which this growth occurs, and from which these variants arise is simplified, and might be considered as partially dedifferentiated. However, this loss of actual structure and function does not at all imply loss of type-specificity or reversion to totipotency; its fate is determined or locked along certain lines of development. The different forms which it assumes, both in appearance and behavior, would be regarded by many as modulations, reversible functional conversions or varieties of expression of cells which have theoretically the same basic composition [116]. If unequivocal evidence could be adduced for the redifferentiation of some of these forms into other cell types, such as into fibroblasts [3, 8, 108], or if it could be shown that "connective tissue" can be induced to form muscle, as claimed by Levander [66], it would mean a change of type referred to as metaplasia.

The macrophage is not generally given status as a differentiated cell type, and is most often regarded as a modulated variant. Nevertheless its formation entails apparent changes in composition as well as disposition, as compared with its cells of origin.

Acquaintance with the various forms assumed by proliferating and "modulating" structures from mature muscle finds practical application in histogenesis of certain soft-tissue tumors. The occurrence in rhabdomyosarcomas of strap and racquet-shaped cells, sometimes with longitudinally or cross-striated parts, are recognized as caricatured rhabdomyoblasts. But spindle-cell, round-cell, and giant-cell forms usually predominate in these tumors. Of particular interest is the so-called granular-cell myoblastoma, a tumor composed of masses of large polygonal cells with small darkly stained nuclei and acidophilic cytoplasmic granules. Since its description by Abrikosoff [1] as a "myoma," its histogenesis has remained in doubt. It has been suggested that it is of fibroblastic [87] or Schwannian [42] origin or, most usually, a neoplasm of muscular origin [57, 61, 83]. Its resemblance to aggregations of histiocytes has led to its designation as a histiocytoma [74]. From what we have seen of the properties of the large, free, round-cells of muscular origin, it seems possible that similar cells compose the myoblastoma.

6. On Regeneration

The regenerative capacities of animals diminish in the course of phylogenetic as well as ontogenetic development. In adult lower organisms up to and including the urodeles, regeneration of whole members may take place; in higher vertebrates regeneration is limited to the repair of tissues. But almost all mature vertebrate tissues, with the signal exception of the neurons, have retained intrinsic ability to grow and regenerate. Lower forms which can regenerate whole members are also capable of nerve cell regeneration. For regenerative regrowth to occur, the cells of a tissue must be able to undergo at least partial apparent de-differentiation or simplification [72].

In the distal limb regeneration of urodeles, the regeneration blastema is made up of de-differentiated cells contributed by all of the injured tissues of the limb [106]. A nerve supply is required for successful regeneration [71], and is necessary both for the de-differentiation and proliferation phases leading to blastema formation, and for the morphogenetic determination of this blastema [96]. In the restitution of muscles from some aggregations of the blastema the cells follow a course very similar to that in embryonic histogenesis [106].

In higher vertebrates the nervous system has lost this morphogenetic control and cannot organize a mixture of different tissues to regenerate a member. Nor has it any influence on the initiation of regeneration, as we saw, although innervation is necessary for the maintenance of regenerated muscle once formed.

It is fitting, then, to ask what in fact does initiate regeneration in higher vertebrates. Growth-promoting substances and "wound hormones" released *in loco* from damaged tissue [6, 17, 78] have been assigned this role. But experiments with wounded tissue cultures (which are themselves regenerating systems) led Fischer to the conclusion that the presence of growth-promoting factors or substances liberated from injured cells cannot be the main reason for regenerative growth [37]; such growth factors serve rather to accelerate a regeneration which would have been initiated without them.

Indeed, the mere reduction of the cell mass of a system without wounding of any kind, such as occurs for example in the depletion of blood corpuscles in plasmapharesis or hemorrhage, is sufficient to motivate a compensatory regeneration. John Hunter spoke of the "stimulus of incompleteness," and Weigert of the displacement of tissue equilibrium; Jacques Loeb [73] epitomized this view in saying that "it is the organism as a whole which represses the phenomena of regeneration in its part, and it is the isolation of the part from the influence of the whole which sets into action the process of regeneration."

The theory of growth and differentiation put forward by Weiss [116] postulates that the cytoplasm of cells contain type-specific "master" molecules which act as templates for the replication of type specific protoplasm. Small-molecular, complementary compounds, which may pre-empt and inactivate the templates and therefore are called "antitemplates," are also supposedly produced as by-products of the modeling process, and may diffuse in and out of cells. Since the rate of formation of type-specific protoplasmic materials (growth) depends upon the number of free templates available, the antitemplates would act to repress growth. According to this view, a reduction in general "antitemplate" concentration consequent on loss of cells which normally produce them would free more templates in surviving cells of the same type for the manufacture of new specific molecules necessary for growth (compensatory hyperplasia). Templates themselves liberated from injured cells, would act as growth-promoting substances by binding extracellular antitemplates in the locus of injury, thus reducing their growth inhibitory effect. This is proposed as a mechanism of local repair. These interesting hypotheses have yet to be informed by specific data for each cell type.

The experiments of Rose [93] on the production of substances by adult tissues which specifically suppress differentiation of like embryonic tissues offers support to the hypothesis of a type-specific inhibitory influence exerted by mature tissues. In general the theories brought together in Weiss' formulation [116] also accord well with the behavior of cells *in vitro* in regard to the influence of transferring, renewal of medium, and growth-promoting factor in stimulating growth, but some observations call for additional explanation. Fischer concluded that removal of waste or metabolic products which would presumably include "antitemplates" cannot be the main reason for the regenerative process [37]. For instance, the outgrowth from multiple fragments cultivated under identical conditions in a plasma medium alone attained a greater area than from a single fragment grown under identical conditions; a fragment inserted into a flask in which cultures had attained maximum size and ceased growing proceeded to grow normally. In these experiments, however, it must be assumed that growth factors from injured cells were carried over with the explants. Nevertheless, these substances may be present in only very minute amount in cultures from washed transplants, even if growth stimulating factors were constantly liberated from dying cells of the outgrowth in culture. The mere introduction of natural or artificial supporting structures such as blood clots, cellulose, gelatin, or polyvinyl sponges into body cavities or tissue spaces, with minimal tissue damage or tissue removal, results in extensive connective tissue repair ("organization"). These manipulations, while they do call forth some inflammatory exudate from which growth substances might be liberated, serve to increase the available surface for expansion. Yet this mainly mechanical increase of surface is an important consideration in explaining the causes of local reparative growth. In culture, too, conditions which prevent the migration of cells, or the occupation of available surface by cells, will inhibit growth in spite of adequate fluid change, good nutritional conditions, and the presence of growth-promoting substances.

We have so far considered only some of the factors which might initiate regeneration and repair. It need hardly be emphasized that in the organism this process is complex and is affected by numerous neural and humoral stimuli, as well as by purely topical influences. In addition, each tissue-type (bone, skin, epithelium, etc.) is unique in its regeneration, with regard to its anatomy, its nutritional requirements, its intrinsic potencies for growth, differentiation, and transformation, and its response to systemic influences such as hormones.

Of the latter, the action of ACTH, cortisone, and related anti-inflammatory steroids have been most studied. The marked inhibition of reparative growth of the "mesodermal" connective tissues by cortisone both *in vivo* [92] and *in vitro* [49] is now well known. The evidence indicates that these steroids act directly on the cell, probably interfering with protein synthesis. It may be that endocrine influences generally act to retard regenerative growth [58]. Discussion of the organismal problems of repair and regeneration, and the behavior of various tissues, may be found in recent works [16, 23].

On a cellular, and ultimately subcellular level, the events of growth and of differentiation, which occur in regeneration, might best be regarded as phases in the unfolding of a fundamentally related process.

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14. *Pathological Swelling and Vacuolization of Cells*[1]

BY ELIAS E. MANUELIDIS

SWELLING OF THE CELLS and formation of cytoplasmic vacuoles occur in many pathological processes. Both are a very common reaction or degeneration form of the cell to a large variety of injurious conditions. Furthermore, swelling and cytoplasmic vacuolization have been described as prominent morphological features in a form of cellular alteration commonly called by pathologists "cloudy swelling." This cellular change has been and still is the subject of long discussion and dispute among cytologists. An excellent review of the topic is given by Cameron [32] and, more recently, Altmann [7].

We are becoming increasingly aware of the limitations of the light microscope in studying morphological changes. A similarity of morphology among different changes does not necessarily imply either identical etiology or nature. Swelling, for instance, may be caused by increase of protein, or intake of extracellular fluid. Vacuoles may be the result of various cytoplasmic alterations such as changes in protein concentration, alterations of mitochondria, and permeability of the cell membrane.

As has been recently emphasized there is increasing need for application of more modern techniques—for example, histochemistry [28, 90]—to the study of pathologic states. With increasing use of such methods as phase-contrast, dark field, and electron microscopy and histochemistry, it is reasonable to expect that more information will be obtained about the nature of processes which, at present, are obscure.

The scope of this paper will be restricted to some of the pathological changes associated with swelling and vacuole formation of cells. In a large variety of cells these alterations are labeled "cloudy swelling." This presentation will not be limited to tissue cells. The formation of vacuoles and the swelling occurring under circumstances injurious to living cells in tissue cultures will also be considered.

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It is the aim here to discuss the results obtained by investigating swelling and vacuolization in cells with the help of more delicate methods. It is considered important not only to present the positive results but to report on many of the major unsolved aspects related to the regressive changes under consideration.

1. Swelling and Formation of Vacuoles in Tissue Cells

CLOUDY SWELLING. Cloudy swelling, also known as parenchymatous, albuminous, vacuolar, or hydropic degeneration, has been described in liver, kidney, muscle, heart, and nerve cells. Not all organs involved present gross findings. The liver and kidneys, however, in classical instances, show increase of weight and abnormal swelling. The tissue bulges when cut and is opaque; the details of its macroscopic appearance are obscured. The early stages of this alteration are characterized by swelling of and the appearance of granules or droplets in the cytoplasm. These granules are soluble in dilute acid and alkali and give a positive protein reaction. Granules, however, are not always described in the affected cells. The nucleus is usually not involved, but may be slightly swollen. In later stages the foamy cytoplasm becomes markedly vacuolated, the nucleus has an eccentric position, and it may reveal lysis or rhexis. Not all of these microscopic findings are recognized by all investigators as necessary features of cloudy swelling. Cloudy swelling is usually thought of as a mild, reversible reaction, which may, however, progress to necrosis if the initial damage is severe.

The controversy about the nature of cloudy swelling is due predominantly to the fact that this cellular response has been examined in a variety of morbid disorders of different etiologies. For instance, cloudy swelling has been described in compensatory hypertrophy of one kidney after surgical removal of the other, in sepsis and in infections, in ligation of the ureter or of the renal vein, in viral infections, in poisoning, and in many other conditions. Bell [17] recognized this state of affairs long ago and suggested that the term "cloudy swelling" be used only in macroscopic evaluation.

It is not surprising, therefore, that some authors called cloudy swelling a degenerative change [18, 68, 72, 102, 146, 151], while others considered it a progressive alteration associated with increased cellular activity [2–4, 49, 132, 136]. Still other investigators have expressed doubts of its existence and have felt that it represented post mortem change, even though experimental workers have produced cloudy swelling under situations eliminating post mortem change. Confusion surrounding the subject is caused by the fact that cytoplasmic vacuoles and cell swelling are phenomena occurring in a large variety of morbid and experimental conditions.

Review of the literature clearly reflects the controversy about cloudy swelling not only among different authors but also occasionally in the ideas expressed by one and the same investigator. Virchow had reported that cells showed swelling, cloudiness, and granules in degenerative processes like parenchymatous inflammation [157, 158]. But under the same heading of cloudy swelling he also classified the cellular changes observed in compensatory hypertrophy of one kidney after loss of the other. In later years he considered cloudy swelling as a more progressive alteration due to increased absorption of soluble material which precipitated within the cytoplasm before being metabolized and thus caused cloudiness of the cells. He thought that cellular swelling was caused by abnormal metabolic substances [159].

As has already been mentioned, in cloudy swollen cells large numbers of fine protoplasmic granules have been observed by many investigators. This accumulation of granules was thought to cause opacity of the cells [132]. The formation of granules was believed to be the result of nuclear disturbances [102] and further to represent degenerative products [151]. In kidney cells after ligation of the renal vein the Altmann granules were found to be decreased [136]. In the liver cells, after poisoning with phosphorus, however, no change of their number has been observed [137]. Other authors believed that the granules observed in cloudy swelling were not identical with those described in normal cells [17, 58, 68]. They were of different structure [68] and larger [17]. In experimentally induced cloudy swelling of the liver by different means (poisons, ligation of hepatic artery and duct) it has been found that the granules disappear rapidly [146].

The granules in cloudy swelling have delicate walls and swell in acetic acid, dissolve in excess acid, and do not dissolve in fat solvents. Further, they are slightly refractile and give a brown discoloration in iodine and a reddish color in sugar solution and sulfuric acid [32]. It has been mentioned that the protein reaction of the granules is positive. Swelling of the cells has been induced with distilled water and with acid in rabbit's liver and renal cells [62] and by mechanical injury of cells [36]; this was attributed to precipitation of protein due to increased cellular acidity [32, 62]. Furthermore, appearance and disappearance of cellular granules in acid has been likened to the precipitation and re-solution of proteins by acid [62].

Zollinger studied the behavior and morphology of the cytoplasmic granules with the phase contrast microscope and felt that these were heterogeneous elements with different functional properties [167]. Although these granules are heterogeneous, their morphology in phase-contrast is similar and they represent intracellular storage of exogenous or endogenous material probably containing protein [167]. Further, it has been reported that these granules in liver cells are composed mainly of phospholipides and ribonucleic acid [40]. Zollinger called this heterogeneous main group "storage granules" and classified it into three subgroups:

(1) The "secretory granules," which were studied in recent years in the liver cells of *Amphiuma tridactylum* by Claude [40].

(2) The "starvation" [96] or "degeneration" granules [101], which were observed in living cells and thought to represent waste products of cellular metabolism. Zollinger [167] found that this type of granule enlarged in suspended living cells and that this phenomenon was dependent upon the temperature of the suspension medium and the length of time of suspension. He further considered the building material of the degeneration granules to be of endocellular origin.

(3) The "resorption" granules resulting from "resorption and intracellular accumulation of a primarily extracellular material." These granules were observed in the renal tubules of experimental animals after intraperitoneal injection of heterologous protein and probably represent resorption of foreign protein from the urine [118]. Further, resorption granules were considered a common finding in human glomerulonephrosis [164].

In which of these three subgroups belong the granules described in cloudy swelling is a question which remains to be answered. It could very well be that the granules seen in cloudy swelling induced by toxic substances are similar to the "degeneration" granules. Those seen in one hypertrophic kidney after removal of the other or those described in some forms of nephrosis [129] may be different and may be classified as "resorption" granules. It is conceivable that more information about the nature of the granules may enable pathologists to differentiate between those changes of cloudy swelling which were thought to be "degenerative" and those cellular alterations which have been labeled "progressive." As it stands now, regardless of some positive findings about the protein reaction of the granules, much remains to be learned about their definite histochemistry and their origin.

Many authors have expressed the view that alteration of the mitochondria gives rise to the formation of cytoplasmic granules [8, 23, 57, 58, 73, 92, 123, 139]. Nevertheless, granules occur all over the cell and not only in the region of the mitochondria. Examination by phase-contrast microscopy of tissues exposed to different solutions revealed swelling of the mitochondria but no transformation into granules [167]. It could be that their absence is due to the particular design of the experiment, namely, that the mitochondria behave in one way when suspended in various media and react differently, with the appearance of granules, in a slowly progressing degeneration induced by other means [7]. No similarity between secretory granules and the microsomes [16] or ultramicroscopic particles [20] has been noticed in experiments with phase-contrast microscopy [167]. However, Claude [40] has expressed the view that mitochondria and secretory granules "constitute extreme forms in a continuous series of cytoplasmic elements." Great chemical similarity between mitochondria and microsomes has been reported [10]. More recent investigations, however, demonstrate marked differences between mitochondria and microsomes of the liver cells [81] and different distribution of enzymes in the microsome fraction and mitochondria of the oyster egg [41].

Long ago it was reported that mitochondria played a very important role in the genesis of cloudy swelling. New experimental data have added support to this view, which is becoming increasingly popular with authors studying cloudy swelling [165].

During the present century numerous reports have accumulated which describe profound changes in mitochondria under various pathological conditions. Thus alterations of the mitochondria have been seen in poisoning and in intoxications [14, 22, 37, 38, 60, 61, 83, 86, 117, 130, 131, 139, 143, 148], in conditions associated with anoxia [54, 105, 116], in inanition [21, 35, 74, 87, 88, 103, 112, 130, 163], in deficiency diseases [27, 50, 51, 116, 148], in abnormal changes of the environmental temperature [11, 88, 104, 111, 134], in infections [24], and in regeneration [119]. It is now accepted that mitochondria are very sensitive to hypotonic and hypertonic solutions, responding rapidly with changes in morphology. This reaction of mitochondria to osmotic changes will be discussed later. Further, it is also well known that post mortem changes can affect these sensitive structures [92, 107, 108, 125, 149]. It is conceivable that some of the early reports describing mitochondrial changes were not very accurate because of these autolytic changes. Furthermore, under normal conditions the mitochondria within the cells of some parenchymatous organs, e.g. kidney, reveal marked variations in distribution, stainability, and number [148].

Regardless of these reflections so many observations have been reported of mitochondrial changes in cloudy swelling associated with various diseases like poisoning, infections, anoxia, etc., that one may consider these alterations of mitochondria as the most constant and prominent deviation in cloudy swelling. Mitochondrial changes seem to be the most important and consistent occurrence in the findings of many authors whose views regarding other morphologic features of cloudy swelling (swelling, granules, vacuole formation, protein changes, and increase of water) deviate widely.

The mitochondria are complex structures composed of protein and lipides concentrated in a superficial zone [19, 20]. The body of the mitochondrion has been reported to contain RNA, while the membrane probably contains DNA and lipides [168]. With Giemsa stain and ultraviolet light the external mitochondrial zone was found to consist of ribonucleic acid [120]. Mitochondria treated with desoxyribonuclease formed confluent larger structures [168]. When treated with ribonuclease they disappeared [168]. Examination of the mitochondria with the electron microscope revealed an external smooth membrane and an internal folded membrane producing cristae or lamellae across the organelle [39, 124, 141]. The morphologic changes of the mitochondria in cloudy swelling consist of transformation of the filamentous structures into granules, spherules, or droplet-like structures. The stainability of mitochondria decreases as these changes progress. Mitochondrial changes in the renal and hepatic cells have been reported in the early literature in infections [92] and of triton and axolotl cells exposed to hypotonic solutions [8].

In cloudy swelling examination of mitochondria with the phase-contrast microscope showed enlargement of the mitochondria and detachment of the membrane from the mitochondrion body; these changes were assumed to be the result of water intake. Furthermore, following intraperitoneal injection of egg albumen accumulation of this foreign protein occurred within the renal mitochondria, which were transformed into brilliant granules [166]. Experimentally produced cloudy swelling by *Salmonella murium* toxin, $HgCl_2$, and the ligation of the renal pedicle in rats, and by diphtheria toxin in guinea pigs reveal the following changes in the nucleic acids. A diminution of DNA only was detected in animals injected with bacterial toxin. These animals revealed the purest form of cloudy swelling produced in this investigation. The nuclei of the proximal tubules stained very faintly with Feulgen stain. A decrease of RNA and DNA was found in the kidneys damaged by $HgCl_2$ poisoning or by ligation of the renal pedicle. This change appeared only in the areas of necrosis, whereas the nuclei of the undamaged cells stained slightly more with Feulgen reaction than normally [65]. Another histochemical study has been reported in lower nephron nephrosis, in which cloudy swelling is a prominent feature. Alkaline phosphatase was absent from the epithelium of the proximal convoluted tubules in scattered groups of nephrons and there was a diffuse decrease of alkaline phosphatase in all the nephrons. Further, no droplets were seen in the epithelium of the proximal convoluted tubules despite consistent albuminuria [110]. No reference is made in this investigation to mitochondrial alterations.

Evaluation of the available information concerning mitochondria reveals that mitochondrial change is a common denominator in a large group of pathologic changes which have been labeled "cloudy swelling" but occurs rarely, if at all, in another group of conditions, such as the neuronal and other cellular changes following viral infection, which have also on occasion been termed "cloudy swelling." It is possible that this fact, coupled with other techniques, may allow a more clear-cut differentiation between these two groups.

Swelling of the cells and vacuolization of the cytoplasm have been related to several factors, of which the intake of water has received the most discussion. In this connection it is emphasized that there are numerous unanswered questions concerning increase of fluid in the cytoplasm, the nature of the vacuoles, and their possible relationship to each other. Recently doubts have been expressed as to whether vacuoles should be considered as a characteristic feature of cloudy swelling [7]. The view was defended that this term should be restricted to those cellular changes associated with structural alterations of the mitochondria [7]. A large number of pathologists believe that hydropic and vacuolar forms of degeneration, though not necessarily identical with cloudy swelling, are nevertheless closely related to it. Both hydropic and vacuolar degeneration are characterized by the presence of many vacuoles. Vacuolar degeneration is classified by some authors as an entity and is separated from hydropic degeneration on the basis of the large number and small size of the vacuoles. Both are encountered in a large variety of altered cells including nerve cells. Yet some neuronal changes associated with vacuolization may occur without obvious alteration of the mitochondria —for example, in viral infections. The question arises whether those cellular changes discussed previously and associated with detectable mitochondrial alterations with or without vacuoles belong to a different category from the cellular alterations revealing pronounced vacuolization without associated changes in the mitochondria.

Many papers in the literature ascribe cellular swelling to alterations in the exchange of fluid between cells and environment. After ligation of the renal artery the presence of vacuoles in the kidney cells has been attributed to imbibition of water [162]. Uptake of water was also assumed to be the cause of the cellular swelling in cases of ischemia of the kidney [152]. Albrecht, adding saline or water to fresh tissue, observed the appearance of fine vacuoles in the cytoplasm. He thought that the normally fluid and homogeneous cytoplasm could be converted by various injurious factors to a suspension of nonmiscible components. This phenomenon was called "tröpfige Entmischung" and was assumed to be the basis of cloudy swelling [2–4].

As early as 1914 Anitschkow described swelling and vacuolization in hypotonic solutions and shrinkage of the cells in hypertonic media [8]. Injurious substances such as narcotics and acids may cause permeability disturbances which are associated with swelling [62, 99, 161]. It has been stated that cells "swell in hypotonic, isotonic and even hypertonic solutions," apparently because of changes in osmotic pressure [32]. In the older literature it has been reported that sometimes swelling of mitochondria does reverse in hypotonic solutions [14]. Furthermore, when pieces of liver from cases of septicemia are soaked in isotonic potassium chloride or sodium chloride solutions, they take up less fluid than material obtained from cases of accidental death [153–156].

In recent years Opie has undertaken an interesting series of studies dealing with the problem of osmotic pressure in cells. In the cytoplasm of cells of liver and kidney, bodies were found which were designated broadly as "cytochondria" and which responded to the water content of the cells. These bodies, some of which were mitochondria, constituted an osmotic system within the cytoplasm. In general they swell following intake of water, although the swelling is modified in a variety of conditions. In frozen liver that has been thawed the cytochondria become swollen, although the over-all size of the cells diminishes. Solutions of isotonic sodium chloride and isotonic potassium chloride delay, whereas isotonic calcium chloride enhances swelling of the cells and the cytochondria. Further, osmotic changes in the cytochondria were found to be reversible [121]. Opie also examined the movement of water in tissues removed from the body and placed in various fluids. The exchange is similar to that which occurs when water passes by osmosis through a semipermeable membrane. The initial movement of water induced in tissues is dependent upon forces which are active during life but which are soon impaired by injury of the tissues. Solutions of sodium chloride isotonic for parenchymatous cells of liver, kidney, and pancreas, have twice, less than twice, and three times, respectively, the molar concentration of sodium chloride of the blood serum [122].

The problem of osmoregulation was attacked recently by a different method and the results obtained are in general agreement with the movement of tissue water mentioned above. Robinson studied the osmoregulation in surviving slices from the liver of rats and found that respiration governs the exchange of water between the normally hypertonic intracellular fluids and the environment. Further, some of the energy released during respiration is utilized through the mediation of compounds containing high-energy phosphate bonds for the transport of water out of the cells. The oxygen consumption of liver slices was reduced when the concentration of the medium was outside of a range lying between 0.20 and 0.45 osm/1. When the concentration of the medium varied between 0.10 to 0.58 osm/1 slices respiring at 38.5° C contained less water than those at temperatures between 1 and 3° C. Inhibition of the respiration by cyanides increased the water content of the liver slices. This phenomenon was reversible and the oxygen consumption was increased when the cyanides were removed. Small concentrations of 2, 4-dinitrophenol increased and larger concentrations reduced the oxygen consumption. Both increased the amount of water in the tissue. Owing to certain common features between the findings observed in this experiment and cloudy swelling, the author suggested that the latter results from a disturbance of the active transport of water from the cells [135].

The results obtained by Robinson are in partial disagreement with recent investigations of Aebi, who studied the oxygen consumption, the water and potassium content of surviving slices of guinea pig liver [1]. The author did not find a close relationship between water content (swelling) and respiration, but did find an inverse correlation between potassium content of slices and degree of swelling. The water content of the liver slices was 710–770 mg per gm fresh weight and potassium content, 90–120 millimoles. In cold, in anoxia, in calcium-free medium, and in medium containing less than 10 millimoles potassium, the normal potassium gradient between the cell and the extracellular fluid could be maintained. Swelling of the cell accompanied the loss of potassium [1]. In this connection it is worth mentioning that long ago Uher [153–156] found in cloudy swelling that the potassium was decreased and the sodium, chlorine, and calcium increased. Eppinger [56] called the loss of potassium and its replacement in the tissue by sodium "transmineralization."

The papers cited above indicate that swelling and vacuolization of the cytoplasm are related to osmosis. The following questions may be asked about the role of the mitochondria in connection with disturbances of osmoregulation and the associated swelling and vacuolization. (1) Are mitochondria altered in different solutions of varying osmotic pressure? (2) Is the swelling of cells secondary to the accompanying mitochondrial changes? (3) Are mitochondrial changes the source of vacuole formation?

The first question can be answered affirmatively by the aforementioned data. Many additional reports in the literature leave no doubt about the marked mitochondrial changes which occur, especially in hypotonic solutions [9, 14, 26, 31, 44, 48, 55, 59, 77, 79, 113, 142, 165, 167]. Biochemical studies of isolated mitochcondria *in vitro* contributed much to the understanding of alterations occurring to these structures in various media [43, 44, 77–79, 127, 128, 142]. In this way valuable information has been obtained about the function of mitochondria under normal and pathological conditions. It has been found that the respiratory activity is associated with the mitochondria in liver, kidney, and heart muscle cells [76, 80, 89]. Harman reported in studies on oxidation by mitochondria, that the integrity of their structure and the maintenance of the cyclophorase complex were interdependent [76].

The sarcosomes (mitochondria) of heart muscle catalyse many intermediary metabolites and the phosphorylation of adenosinediphosphate to adenosinetriphosphate [43]. The effect of tonicity of the medium on the respiratory and phosphorylative activity of heart-muscle sarcosomes was investigated by Slater and Cleland [142]. These investigators found that the α-ketoglutaric oxidase system has a pH optimum at 7.4 and its maximum activity is obtained under hypotonic conditions. As the tonicity of the medium increases, the activity of the α-ketoglutaric oxidase decreases. High concentrations of phosphate decrease the P:O ratio; this is, however, unchanged in a wide range of sucrose concentrations which have a marked effect on the activity of α-ketoglutaric oxidase system. Hypertonic sucrose is an inhibitor of the succinic oxidase system [142].

Harman and Feigelson in careful investigations correlated the function of mitochondria *in vitro* with cytological examinations [78]. The findings of their investigation are given below. Freshly isolated mitochondria in 0.50 and 0.80 molar buffered sucrose are elongated rodlets. In more dilute sucrose (0.25 and 0.31 molar) the mitochondria are swollen, spherical, dense, and homogeneous. A small proportion of mitochondria, however, revealed in this dilution a white "target" refractile area surrounded by a peripheral dense zone. A few persistent rods were among these "spherical-dense" and "target" forms. With further dilution of the medium the authors observed more swelling of the mitochondria, which "become altered internally," either into a hemispherically segmented light and dark zone called a "limbus" or into a dense marginal eccentric "crescent" with a large clear zone. The requirements for magnesium were found to be very high in determining the optimal conditions for oxidation of intermediates of the citric acid cycle. "Maximum oxidation is attained at a magnesium level of 10 micro moles per ml and is associated with a high concentration of total dense forms and 'target' type mitochondria." On the contrary in the flasks containing low concentrations of magnesium, the "crescent" is the prevalent form. Suspensions of mitochondria in various concentrations of sucrose in an optimal concentration of magnesium were examined microscopically before and after the oxidation period in Warburg vessels. In discussing their results, Harman and Feigelson stated that "obliteration of the internal structure of the mitochondria accompanied cessation of cyclical oxidation" and that "several states of the mitochondria affect and appear to govern the rate of oxidation." Rod forms of mitochondria allowed a slow oxidation and "crescent" forms revealed a diminished capacity to metabolize. The authors conclude that the "crescent" type of mitochondria characterized cloudy swelling [78]. The experimental data of Harman and Feigelson have been given at some length, because they demonstrate the value of an investigative approach which combines study of induced morphological changes with biochemical alterations of function.

Mitochondrial changes in hypotonic solutions are referred to as "osmotic swelling" as opposed to the "nonosmotic swelling" induced by ADP, AMP, phosphate, and pyrophosphate. The mechanism of the nonosmotic swelling is not clear [127, 128]. Raaflaub found adenosine triphosphate to be "a powerful inhibitor of the nonosmotic swelling" of mitochondria, but to have no influence on the "osmotic swelling" of these organelles [127]. The author considered mitochondrial swelling reported by others [120a, 166] in cloudy swelling and developed the hypothesis that these changes were due to intracellular decrease of ATP [128]. Previous to the above investigation, Cleland reported diminished permeability to potassium chloride of sarcosomes (mitochondria), when adenosine triphosphate was added to the medium. Hypotonic swelling of the sarcosomes was not influenced by this addition [42].

It has been reported that transformation of the mitochondria from rodlets into other types characterized by swelling, enspherulation, and "crescent" formation is associated with enhanced ATP-ase activity [79]. Dinitrophenol, however, has "no action on native rodlets" and can activate mitochondrial ATP dephosphorylating mechanisms "in restricted ranges of osmolarity." This activation is proportional to the ability of the compound to transform the enspherulated mitochondria into crescents [85]. Dinitrophenol is known to cause inhibition of respiration and phosphorylation mechanisms [98, 140].

All the above interesting and valuable experimental data about the mitochondria indicate a very complex and important function of these organelles in the cells, and the results of Harman and co-workers indicate a close interdependent relationship between structure and function.

The nature of the swelling of the mitochondria is not yet clear. A critical consideration of this problem has been given recently by Harman and Kitiyakara [79]. These authors considered several varieties of swelling. One is associated with autolytic or thermal distention of the organelles which is inhibited among other factors by addition of ATP (probably corresponding to the already mentioned "nonosmotic swelling" of Raaflaub) and another corresponding to the "osmotic swelling" (Raaflaub) induced in hyposmotic conditions. The authors do not regard the existing differences between the two categories as real and think that the mechanism of the swelling in both is similar. In this connection, it is worth mentioning the results of Cleland and Slater [44] that in "nonosmotic" transformation of mitochondria complete disintegration does not occur; instead the mitochondria aggregate in clumps. Further, Harman and Kitiyakara deny that mitochondria are semipermeable like the plasmalemma, although they admit the probable existence of a surface interface [79]. Harman in a previous investigation regarded the mitochondria as "tightly coiled or compact gels" [77]. Whether a differentiation between altered mitochondrial forms is possible as some investigators believe [44, 127, 128], remains to be answered. If this differentiation is possible, then the alterations of mitochondria will probably facilitate differentiation between the several cellular changes labeled as cloudy swelling.

To answer our second question, whether the swelling of the cells is secondary to accompanying mitochondrial changes, there are indications supporting the view that the swelling of cells which occurs in many pathological conditions accompanied by cloudy swelling is the result of mitochondrial changes or at least associated with mechanisms set in motion by mitochondrial disturbances. However, before a clear-cut answer can be given to this problem, it is necessary to know whether the function and structure of mitochondria are really disturbed in all those pathological processes classified in the main group of "cloudy swelling." It is conceivable that the swelling of the cells which reveal "resorption granules" (see page 420) is due primarily to resorption of extracellular material and not to direct mitochondrial change. Furthermore, the swelling observed in neuronal degeneration in some viral infections—for example, poliomyelitis—is probably independent of mitochondrial changes, since these organelles have been reported to be unaffected.

It cannot be denied, especially in the hypotonic form of degeneration, that the mitochondria are foamy and reveal a marked degree of vacuolization. With the data now available the question of mitochondrial origin of vacuoles cannot be answered. There is some evidence, however, pointing in that direction. Cameron and Abraham [33] cited ultra-centrifugation by Judah, Christie, and Rees [84] of damaged liver cells revealing hydropic changes which pointed to mitochondrial origin of the isolated vacuoles. Nevertheless, doubts have been expressed about the mitochondrial origin of the vacuoles, and it has been emphasized that the vacuoles occurring in hydropic degeneration are morphologically different from those observed in cloudy swelling. In the latter the cells have a foamy appearance whereas in the former the vacuoles are clearly seen under lower magnification and are delineated by membrane-like structures [7]. Furthermore, degenerative nuclear changes are the rule in hydropic degeneration whereas the nuclei in cloudy swelling are usually not significantly altered. In experimental hypoxia vacuolar degeneration of cells has been reported [5, 6, 30]. The observed vacuoles have been interpreted as a vital production of the cell [5, 6]. No correlation of the mitochondria with vacuolization of the cells is given in the above references. In addition, it is known that occasionally swelling and marked vacuolization of the cytoplasm can occur in cells without detectable mitochondrial changes. Reference is made to the neuronal degeneration accompanying viral infections. In poliomyelitis no changes of the mitochondria have been described [25, 109] in the cells before the stage of acidophilic necrosis is reached. In herpetic encephalitis characteristic nuclear inclusions or extensive vacuolization of the neuron may occur, yet the mitochondria are apparently unaffected [46]. Apparently in this neuronal form of degeneration other mechanisms than the degeneration of mitochondria are responsible for the vacuolization of the cytoplasm. The basophilic material (RNA) disappears. Present information suggests that the nucleoprotein apparatus of the cells is markedly affected, as studies of the development of rabies virus in the central nervous system of chick embryos have indicated [144].

Much more work is needed in this field before one can draw a definite conclusion about cytoplasmic vacuoles. Some of the droplets seem to develop on the basis of mitochondrial alterations; however, this may not be the only mechanism which is implicated in cytoplasmic vacuole formation.

There is much confusion in the literature regarding the protein changes observed in cloudy swelling. Coagulation [45] or precipitation [23] of protein have been described in the cytoplasm of cloudy swollen cells. It has been thought that the opacity of the cells in turpentine poisoning and acute yellow liver atrophy is caused by changes in the protein molecules [18]. Recently, in agreement with earlier work, intracellular protein was found to be increased in cloudy swelling [126, 150]. Swelling has been attributed by some to be due to the affinity of proteins for water [32]. Hoppe-Seyler suggested the name "albuminous swelling" instead of "cloudy swelling" because he found an increase in protein in this cellular change [82]. Nevertheless, the majority of authors do not regard the protein changes associated with cloudy swelling as an essential feature of this condition. By no means is protein always increased in cloudy swelling [52, 67, 70, 153, 160]. In the protein metabolism of cloudy swelling much more investigation is required before definite conclusions can be drawn. Future investigation of protein changes may give the answer to this problem. It may also permit differentiation of those cellular alterations which were thought to be *progressive* from those which were thought to be *degenerative* changes of cloudy swelling, both included in the main group of cloudy swelling.

In early work on cloudy swelling, fatty changes in the cytoplasm of the affected cells were reported. It appears now, however, that fatty degeneration is neither a generally recognized characteristic change nor a consistent feature of cloudy swelling.

It seems likely that cloudy swelling precedes fatty degeneration of the cell and probably depends upon the severity of the damage, the duration, and the nature of the causative agent. Fatty degeneration has been reported to be associated with cloudy swelling [18, 23, 132]. In fatal carbon tetrachloride poisoning of humans, severe cloudy swelling with granularity of the cytoplasm and the appearance of small amounts of fine fat droplets have been described [114]. The same injurious factors may induce both cloudy swelling and fatty degeneration [143]. Degeneration of mitochondria has been thought to give rise to fat droplets [47, 54] or to cause changes in the lipide metabolism [64]. In experimental cloudy swelling, the oxidation of butyric, crotonic, and valeric acids was diminished, whereas that of the β-hydroxybutyric acid was unchanged. These metabolic disturbances were associated with considerable alterations of the mitochondrial structure [64]. Fat droplets within the mitochondria of degenerated liver cells have been reported [87]. Another study of the same material, however, revealed vacuolization and the disappearance of mitochondria without the formation of fat [53]. Further work is necessary to establish the role of mitochondria in fatty changes. In studies with the phase contrast microscope, no fat droplets have been detected in the swollen mitochondria [167]. It could be that the alterations of the mitochondria cause a disturbance of the general lipide metabolism of the cell rather than a direct transformation into fat droplets. This remains to be seen.

SUMMARY OF CELLULAR CHANGES IN TISSUES. In summarizing the reported findings concerning cellular swelling and cytoplasmic vacuolization, particularly the cellular changes associated with cloudy swelling, only noncommittal comments should be made. The topic is still subject to dispute and the investigative results obtained in recent years, although very encouraging, do not as yet answer the many problems associated with the cellular alterations under consideration.

The nature of the cytoplasmic granules seen in cloudy swelling is not clear. Additional information obtained in recent years indicate that although they reveal a similar morphology, they are of heterogeneous origin and they apparently represent intracellular storage of exogenous or endogenous material probably containing protein. Whether the granules arise from degenerated mitochondria is not yet established, although some information indicates such a possibility. The relation of the granules to the microsomes is still in dispute.

Very valuable and important data have been obtained by investigations of mitochondria *in vitro*. Their function seems to be closely related to their structure and alterations of the latter necessarily affect the former. Valuable information has been obtained recently concerning the enzymatic, oxidative and phosphorylative activity of the mitochondria. As it now stands, it seems that in a variety of pathologic conditions belonging in the main group of cloudy swelling, the mitochondrial changes constitute a prominent morphological feature and their altered function affects, directly or indirectly, cellular function and morphology. There are data suggesting different types of mitochondrial changes, yet additional work is necessary before this conclusion can be accepted. Predominant changes of the mitochondria in some pathologic conditions belonging in the group of cloudy swelling may enable the pathologist to differentiate between these and other cellular alterations in which the mitochondria do not seem to be involved.

Swelling of the cells has been related to several factors, of which the intake of water has received the most attention. Osmoregulation seems to govern the exchange of fluid between the normally hypertonic cell and the environment. Doubts have been expressed as to whether disturbances of respiration may cause cellular swelling, and it has been reported that loss of intracellular potassium is accompanied by intake of water and swelling. Further, the swelling of the cells has been related to the mitochondrial changes among other factors, and to mechanisms associated with their disturbed function. It seems, however, that in some cells swelling is due to other mechanisms than those just mentioned.

The nature of the vacuoles and the problems associated with their formation have been outlined during the present discussion. In some instances at least their formation is related to mitochondrial disturbances and the intracellular intake of fluid. Nevertheless, there are probably other mechanisms responsible for their genesis.

The disturbances of protein and lipide associated with the formation of vacuoles and cellular swelling have been considered and found to be neither constant nor prominent features of the cellular changes under consideration.

Whether vacuolar or hydropic degeneration represents an advanced stage of cloudy swelling or whether the latter is an obligatory precursor of hydropic degeneration remains to be answered.

It is possible that further investigation will allow a decision as to whether cloudy swelling is a primary phenomenon followed by intake of water, protein changes, and alterations of oxygen intake or whether these latter changes are themselves initiating mechanisms for cloudy swelling [71, 106].

2. *Tissue Culture Cells*

As in tissue cells, the occurrence of vacuoles in the cytoplasm and the swelling of the cells observed under certain pathological conditions will be discussed in tissue cultures. Tissue cultures are at the present time being increasingly and successfully used in virological investigation, and the observed cellular alterations in the presence of the virus will receive the most prominent treatment.

It has been reported that the "ground cytoplasm" in growing cells appears optically empty by dark field illumination, which is considered an ideal method for the examination of living cells [100]. Further, a few refractile fat droplets and a few granules staining with neutral red can be seen in the cytoplasm of healthy appearing fibroblasts [100]. The most conspicuous structures, however, which have been observed in tissue culture cells are the filamentous mitochondria, which react under injurious circumstances by breaking down into fine granules before they disintegrate completely. Ludford observed aggregation of the mitochondria around the nucleus as an initial sign of adverse conditions [101]. As in tissue cells, the mitochondria swell and become vesicular under pathological circumstances. There are many reports in the literature describing mitochondrial changes of tissue culture cells in a large variety of pathologic conditions [29, 63, 91, 93, 94, 100, 133, 145, 147]. Recently the action of detergents and other injurious substances has been investigated with the phase-contrast microscope [66]. In the mitochondria of living cells injured by the presence of detergents two phases of the reaction have been detected. During the first phase, the filamentous mitochondria change into a beadlike string followed by fragmentation into grains. In the second phase a small central gray area appears, which enlarges rapidly, becomes clear, and is surrounded by a black irregular zone. In the central clear area gray bands have been seen. The altered mitochondria may expand suddenly into a vesicle. During the second phase signs of cell death have been observed [66]. The above mitochondrial changes were continuously registered cinematographically. In the same investigation the action of dinitrophenol upon living cells was studied. This stimulates active movement of the mitochondria, which become thicker and longer. The nucleoli are more "actively deformed." After this phase of activation the mitochondria become abnormal in appearance and the nuclei shrink and become porous [66].

As in tissue cells, the mitochondria in tissue culture cells seem to be very sensitive structures, reacting rapidly under adverse conditions. In the above-mentioned investigation movements of the mitochondria in the cytoplasm were observed, and were attributed "to metabolic exchanges between mitochondria and cytoplasm" [66].

Ludford cited personal observations and results obtained by Kater [87] indicating the possibility of formation of fat droplets within the mitochondria. On the other hand, he mentioned the observations of Duthie [53] describing marked mitochondrial changes culminating in ultimate disappearance of the organelles without formation of fat.

In the cytoplasm of cells revealing cloudy swelling, granules were detected. The nature of these, as already mentioned, was not completely clear. Granules which stained with neutral red were few in number in healthy fibroblasts and their number increased with adverse circumstances [95, 100]. On the one hand, they have been considered to be manifestations of cellular degeneration [97, 101]. On the other hand, Carrel and Ebeling have interpreted the granules as manifestations of increasing cell growth [34].

Vacuoles and fat droplets have been described in tissue culture. Cytoplasmic vacuolization may be very marked, and the vacuoles were considered by Lewis to be products of autolysis [96].

With increasing use of tissue culture in viral investigations, reports of changes observed in tissue culture cells infected with viruses are becoming more numerous. Swelling of cells, appearance of granules, and vacuole formation in the cytoplasm have been described as morphologic features in growing cells infected with poliomyelitis virus [15, 75, 91]. Unpublished data of the author will be utilized to illustrate the occurrence of these pathologic manifestations. It should be emphasized that the described changes are not necessarily direct cellular responses to the presence of virus. As already mentioned above, vacuolization and granulation of the cytoplasm may occur under the influence of injurious substances other than virus. In tissue cultures of fibroblasts growing from testicular tissue of monkeys and infected with 100 $T.C._{50}$ doses [2] of poliomyelitis virus, swelling of the cells was one of the first detectable signs of cell injury and occurred as late as twenty-four hours after inoculation with the virus. In addition, the cytoplasm had a foamy appearance. Tissue cultures fixed in Regaud's solution and stained with Masson's trichrome stain (modification of Pappenheim), showed that the tonoglia or fibroglia of the cells was markedly decreased in the infected cultures as compared with the controls (Fig. 224). In this connection, it is worth mentioning that the tonoglia or fibroglia is known to be composed of protein and does not have a fibrillary structure like collagen.

The fibroblasts which normally grow in streams and bundles become separated from one another soon after inoculation and their cytoplasm assumes a moth-eaten appearance with protoplasmic projections (Fig. 225). At this stage of infection, healthy fibroblasts may be found next to altered cells. With the appearance of swelling, the occurrence of granules and the appearance of vacuoles were noticed in the infected tissue cultures. Some fine vacuoles were observed in the noninfected tissue cultures and were present also after inoculation. These droplet-like structures stained with Sudan black and were interpreted as lipides. In the infected tissue cultures, however, many fine vacuoles occurred progressively during the process of cellular alteration. Some of these were difficult to differentiate microscopically from the above-mentioned sudanophilic structures, whereas others were large and had irregular outlines. These types of vacuoles did not stain with Sudan black, Sudan IV, and Nile blue sulfate. When stained for glycogen, they failed to reveal a positive reaction. Often the vacuoles were associated with granules and were observed to displace or to cause a concave indentation of the nucleus (Fig. 226). The question arose as to whether these vacuoles and granules were replacing the disintegrating nucleus; however, Figure 226 shows that the nucleus is hypertrophic and, although altered, it is by no means in process of karyolysis or karyorhexis. Its displacement, therefore, may be considered as secondary to the cytoplasmic changes. Very often the vacuoles become considerably larger and occupy large portions of the cytoplasm (Fig. 227).

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[2] $T.C._{50}$ is the dose of virus which produces cytopathogenic changes in 50 per cent of the inoculated tissue cultures. In this experiment 100 × this dose was inoculated in the tubes.

In another tissue culture experiment, vacuoles were produced by another means in order to compare them with the cytoplasmic vacuolization occurring in tissue cultures infected with poliomyelitis. Tissue cultures were infected with the supernatant fluid of human stool, in which no poliomyelitis virus was found. The tissue culture tubes inoculated with this fluid revealed a marked cytoplasmic vacuolization, leading ultimately to complete disintegration of the cells. The vacuoles were much larger than those found after poliomyelitis inoculation, and the fibroblasts were not detached from one another, as was the case after inoculation with poliomyelitis virus, but formed islands of vacuolated cells (Fig. 228). These cultures stained with Nile blue sulphate and showed a pinkish discoloration of the vacuoles. This was interpreted as due to the presence of neutral fat.

Early in the infection with poliomyelitis virus, many small granules were seen in the cytoplasm, often forming large accumulations. These granules, especially in the aggregates, were often basophilic in hematoxylin-eosin stain. As mentioned, they were often associated with vacuoles (Fig. 229).

With progression of the cytoplasmic changes, the nuclei were affected, and some twenty-four hours after inoculation of the tissue culture with poliomyelitis virus, they were slightly distorted and swollen, finally becoming pyknotic and disintegrating. Some forty-eight hours after inoculation much irregular basophilic debris was seen (Fig. 229). This material gave a positive Feulgen reaction and was considered to be the end result of nuclear breakdown. Nevertheless, apparently well preserved nuclei were seen in the presence of well developed cytoplasmic degeneration. Even in cultures revealing marked cellular disintegration and nuclear breakdown, healthy fibroblasts could be observed among the debris; many of these revealed mitotic figures (Fig. 230). The mitotic figures of these remaining cells were counted and it was found that the mitotic activity of tissue cultures inoculated with poliomyelitis virus persisted into the late stages of disintegration of fibroblast colonies.

Many cells revealed a pale cytoplasm, others a slight basophilic discoloration. Tissue cultures were stained with methylene blue, at a pH of 5, in order to determine the behavior of RNA. Infected and noninfected cultures of fibroblasts were incubated at 37° C in ribonuclease and served as controls. Twenty-four hours after inoculation, the poliomyelitis-infected tissue cultures were found to be more lightly stained with methylene blue than the corresponding cultures of apparently healthy fibroblasts. This was thought to be due to a decrease of RNA in the infected cells. Ribonuclease abolished the basophilia of both infected and noninfected cultures. Preliminary studies with alkaline and acid phosphatase failed to reveal any activity when glycerophosphate was used as a substrate. Forty-eight hours and more after inoculation of poliomyelitis, tissue cultures showed marked cellular and nuclear disintegration, which progressed up to complete disintegration of the culture.

In poliomyelitis-infected tissue cultures there are many other interesting cytological findings, which are somewhat removed from the present topic and will be discussed elsewhere.

Vacuolization and appearance of granules in the cytoplasm have been reported in all the studies of tissue cultures infected with poliomyelitis [15, 75, 91]. In the previous discussion of swelling, vacuolization, and appearance of granules in the cytoplasm of tissue cells, the phenomena were considered in relation with mitochondrial changes. Consideration will now be given to cells infected with viruses with reference to the behavior of the mitochondria under these circumstances. Ludford described the appearance of small vacuoles in the cytoplasm of cells infected with fowl pox virus. The mitochondria at this stage of alteration were normal in form [101]. Abnormal mitochondria have been described in electron micrographs of cells infected with mammary tumor inciter, in some cases of mouse hepatitis, and in chick embryos infected with herpes virus [12, 13]. Changes of the mitochondria and association of the organelles with virus particles, however, have not been seen in electron microscopic studies of cells infected with vaccinia and fowl pox viruses [115], or with vaccinia, ectromelia and molloscum contagiosum [69]. In this connection, Ludford described in vaccinia infection both large and small vacuoles, the former containing minute granules [101]. Available information concerning the behavior of mitochondria in tissue culture cells infected with viruses is as yet sparse.

SUMMARY OF CELLULAR CHANGES IN TISSUE CULTURES. As in tissue cells, vacuoles, granules, swelling of the cells, and mitochondrial changes are seen in tissue cultures under adverse conditions. Here the same problems arise concerning the nature of these changes just as in the case of cells in tissues. The information currently available indicates that the vacuoles and granules occurring in cells attacked by a variety of viruses do not seem to be related to mitochondrial changes, as several studies with the electron microscope have shown. Concerning the behavior of protein in viral infections of cells not enough examinations, such as the already mentioned study of Sourander [144] with rabies, have as yet been undertaken. To what degree the vacuoles, granules, and cellular swelling are related to disturbances of nucleoprotein due to the action of viruses remains to be seen.

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15. Cellular Reaction during Virus Infections

BY WILLIAM H. GAYLORD, JR.

LIVING CELLS constitute the only environment suitable for virus multiplication. No synthetic substrate has been devised to replace a cell, nor will virus numbers increase in a dead cell. Although intracellular parasitism is not restricted to the viruses, there seems to be a fundamental difference between viruses and those intracellular parasites which are, in themselves, cells. The higher forms multiply by fission, budding, or sporulation and often they can be cultured extracellularly. Under such circumstances, the role of the invaded cell is that of an almost accidental or passive host, supplying all of the requirements for growth but little else. During the course of virus infections a host cell apparently contributes more than simple nutriments. It may actually participate in the production of virus particles and it is, by contrast, an active host.

The strange tendency of a cell to aid in its own demise has excited the interest of cytologists and virologists alike for some time, but it is still not well understood. One obstacle to more rapid progress in this regard is the fact that the viruses constitute heterogeneous groups of agents. It is often difficult to know whether information gained from one group can be applied to another. In fact, it is reasonable to expect separate and distinct mechanisms to operate where visible manifestations are dissimilar, such as morphological differences in elementary bodies or the production of nuclear instead of cytoplasmic inclusion bodies. Furthermore, the interaction of host and parasite can vary with the host as well as with the virus. A virus which is the causative agent for tumors in chickens, for instance, will cause necrotic liver lesions when it is put into a duckling [21]. Presumably, the mode of reproduction of the virus is unchanged, the difference being one of degree. That is, perhaps young duck liver cells are better virus producers than chicken tumor cells or less resistant to the adverse effects of the virus.

If a given virus can stimulate cells to divide in one tissue and kill them in another it is not surprising to find that closely related viruses may produce dissimilar lesions and cytopathic effects. Conversely, totally unrelated viruses may mimic each other to some extent. Intranuclear inclusion bodies have been described, for instance, in tissues infected with the viruses of variola, one of the largest, and of poliomyelitis, one of the smallest. It follows, therefore, that cytopathology and virus multiplication are not directly related. If, then, each type of virus is reproduced in a fixed manner, the invaded cells must, then, respond according to a highly complex set of stimuli.

1. Cytoplasmic Viruses

THE PSITTACOSIS GROUP. Members of this group of intracellular parasites have long been known as the "largest viruses." They are not now regarded as viruses but they have been included in this discussion because of their history. Virus terminology has been retained for the sake of simplicity. Psittacosis virus elementary bodies are about 280–300 mμ in diameter and, therefore, visible with a light microscope. In 1932, Bedson and Bland [8] described a series of spheres in the cytoplasm of infected cells ranging in size from that of the elementary bodies to some as large as one micron. Bland and Canti [11] later showed by means of infected tissue cultures that elementary bodies were formed from the larger spheres and they suggested that the virus of psittacosis has a complex life cycle. In 1939, Gey and Bang [30] showed that the virus of lymphogranuloma venereum multiplied in vesicles of cultured cells and that pairing of the virus particles was common.

Other cytoplasmic inclusions have also been described over a period of years and one can find reference to inclusion bodies, initial bodies, residual bodies, granules, plasmodia, morulae, clusters, plaques, vesicles, and a host of others in addition to the large and small spheres. It is obvious that the life cycle of the psittacosis group of pathogens involves several compound structures and that some of them are known by more than one name. In most instances, a single name probably has been applied to that which was actually a mixture of cell products and virus. In discussing this point, Van Rooyen and Rhodes [63] cite Halberstaeder's recognition of three distinct parts in an inclusion body as seen in the cytoplasm of a cell infected with trachoma virus. These are: (1) the parasite, (2) a structureless matrix in which the elementary bodies were embedded, and (3) a "plastin-like" substance derived from the cytoplasm. Halberstaeder postulated in 1912 that the "plastin" substance represented a defense mechanism of the host cell as well as the nutritive medium used by the parasite.

The three-component inclusion body is an almost perfect description of that now recognized for some viruses [29], but curiously enough, not for the psittacosis group which includes trachoma. At least, in the only electron microscope study to date of the intracellular morphology of a member of this group (meningopneumonitis virus) the pattern of development was somewhat different [27]. In this study of inclusion bodies in the cells of chick chorioallantoic membrane, a "plastin-like" substance was indeed visible as dense, osmiophilic deposits in the cytoplasm. It was amorphous, irregular, and apparently cellular rather than viral in origin. The intense osmiophilia suggests that a lipide component was present.[1] The material was present in large amounts and in many cells of an early lesion, but it seemed to disappear as the amount of virus increased. Halberstaeder may have been correct in assigning a protective function to his "plastin," but it seems unlikely that it is a source of nutrient because virus development can take place without it. In all probability, Kurotchkin, *et al.* [38] were removing this material when they showed that large forms of lymphogranuloma venereum could be dissolved in ether without affecting the elementary bodies.

No sign of a structureless matrix was apparent in the meningopneumonitis study, but instead, the virus particles were always seen in discrete cytoplasmic vesicles[2] (Fig. 231). There were two or three small vesicles containing a few virus particles in some cells. In other cells a single, large vesicle, packed with virus, almost entirely replaced the cytoplasm. The vesicles were always surrounded by a membrane and, occasionally, mitochondria were flattened against the outside of it, a position suggesting that the vesicle merely pushed the cytoplasm aside as it enlarged.

The entire process of virus reproduction apparently occurs within the confines of a vesicle. Structures seen there correspond to elementary bodies and to the spheres described by Bedson and Bland. The appearance of numerous binary and yeastlike budding forms of the spheres (especially in fibroblasts) indicates that the agent of meningopneumonitis can probably divide by fission at one stage. The smaller elementary bodies would, then, correspond to spores. In other cells of the chorioallantoic membrane, budding forms were less common, while spheres with larger diameters were frequently seen. The larger spheres resembled sporozoa or those of fungi which form multiple endospores, and it is possible that multiple endosporulation is an alternative reproductive mechanism.

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[1] The term "plastin" has been retained here in order to avoid the use of more specific terminology. Nearly identical deposits are encountered in uninfected macrophages and in other infections. Although similar, the various examples could be chemically distinct.

[2] Although the vesicular fluid might be fixed and stained by some methods, the term "matrix" is being reserved in this discussion for a structure seen in vaccinia inclusions.

The course of cellular infection with any virus of the psittacosis group undoubtedly follows a pattern close to that of meningopneumonitis. When the sporelike elementary bodies invade or become ingested by a cell they are rapidly surrounded by an electron dense "plastin" substance in the cytoplasm. If this material is viricidal, or inhibitory, then, in many cases, a cell might recover. If the invading "spore" germinates, the ensuing events apparently depend more on the host than the parasite. When the host cell is a fibroblast, very little "plastin" is produced. The "vegetative" virus which arose from the "spore" now starts to divide by pinching off in the usual manner of schizogenesis. The host cell reacts by laying down a thin capsule around the developing "colony," thereby causing it to be contained in a vesicle. The fact that one cell may contain two or three vesicles means that multiple infection is common, or, that occasional parasites escape from the vesicle of their origin and create new foci of development.

When an ectodermal cell or macrophage is invaded, a different series of events takes place. Generally, more "plastin" is produced, but Levinthal pointed out in 1935 [39] that the nutritional state of a cell has an influence on virus development and, by inference, on "plastin" production. He found that healthy cells reacted to form inclusion bodies which he considered defensive for the cell. The inclusion bodies disappeared when the virus multiplied to such an extent that a cell was overwhelmed. When an injured cell was invaded, lethal numbers of virus particles were formed more rapidly and the amorphous material was never produced. Whether the environment within an ectodermal cell differs from that of a fibroblast because of the ability to accumulate "plastin" or some other reason is difficult to say, but the mode of parasite reproduction is also changed. Instead of splitting in two when they are about 500 mμ in diameter, the vegetative forms seen in ectodermal cells continue to swell until the diameter measures as much as a micron or more. Multiple granules appear within forms of this size and the familiar spore-like elementary bodies can be seen scattered in the same vesicles. Apparently, a type of multiple endosporulation takes place instead of binary fission, but evidence bearing on this point is scarce.

Single, large vesicles are more common in ectodermal cells than multiple small ones. The vesicles become so large that the entire cytoplasm is displaced and the nucleus is pushed to one side. When a cell eventually dies, the parasites remain entrapped within as spores, as if host cell death is a signal for sporulation to take place. Exhaustion of the host is not the only cause, however, for the spores (or elementary bodies) are seen in small vesicles occasionally, and in large vesicles frequently. Compressed, dead cells enveloping a myriad of elementary bodies are the end-product of an infectious cycle as seen in an egg membrane exposed to the air. That virus particles escape to infect other cells should be a foregone conclusion, and yet, if escape be effected it must be on a very small scale. No particles of virus were found outside of cells and no cells were found in a state indicating that they might rupture or burst. The cytopathology might be thought of as debilitation or malnutrition with cell death coming only after a long struggle against an invading semi-dependent parasite. How generalized this conclusion may be remains questionable. The psittacosis group is large and the viruses included in it are responsible for a variety of lesions.

THE POX GROUP. Much of our present knowledge concerning the cytopathology of virus infection stems from studies of the pox viruses. Historically they occupy an important position and the literature surrounding the subject of pox-virus inclusion bodies is both voluminous and controversial. Guarnieri [31] described the inclusion body of smallpox in 1892 shortly after the discovery of the protozoan nature of the malarial parasite. It is generally assumed that he was influenced by that event and, as a consequence, he concluded that the agent of smallpox was a protozoan which he named *"Cytoryctes variolae."* Guarnieri's bodies can be large enough for unicellular animals and he mentioned amoeboid movement in his description of them. There must have been reasonable grounds for doubt, then, when such acute observers as Buist [14] and Paschen [49] described specific particles of a size approaching the limit of resolution for light microscopy. Although these smaller particles are now conceded to be the disease agent it should be born in mind that their true nature is not known even now. Also, the larger inclusion bodies are no longer thought of as protozoa but their real position in biology is still debatable after a half century of investigations.

Basically, the question yet to be answered is one of quantitation. That is to say, how much of an inclusion body is virus, how much is cellular in origin. Cytologists, of course, recognize many kinds of cellular inclusions but in the study of virus infections the term "inclusion body" has assumed a special and sometimes misleading connotation. Inclusion bodies are, in fact, pathognomonic at times because they occur invariably in some diseases. This does not mean, however, that the inclusion body of one disease is necessarily the same as the inclusion body of another. The related viruses of molluscum contagiosum, vaccinia, and ectromelia provide a good example. They are all known to form eosinophilic inclusion bodies in the cytoplasm. The Henderson-Paterson body of molluscum is actually a consolidated mass of elementary bodies with little or no cytoplasm remaining in its host cell. The Marchal body of ectromelia is a limited area in the cytoplasm where virus development is going on. It is rich in a dense material like that previously referred to as "plastin" and contains almost no "mature" virus or elementary bodies. Later in the course of infection the cell does become filled with mature virus and "plastin" disappears, but the molluscum cells never seem to go through such a phase. The two inclusion bodies, therefore, are quite different from each other. The Guarnieri body of vaccinia is the same as the inclusion of ectromelia, except for one thing—these inclusions cannot always be demonstrated even when virus is known to be present. Vaccinia virus induces the formation of inclusion bodies in the corneal cells of a rabbit (Fig. 232), for instance, but not in chick chorioallantoic cells [29]. Although cells are infected and the cytoplasm is definitely more dense in the area of virus development (Fig. 234), the very dense "plastin" is missing. Under these conditions viral development is more like that of molluscum but in a limited zone instead of diffuse in the cytoplasm. The change in behavior of vaccinia cannot be accounted for on the basis of the host involved for ectromelia virus produces inclusion bodies in egg membranes similar to those of vaccinia in the cornea. The situation appears to be similar to the results of Begg, Fulton, and Van den Ende [9], who studied the effects of adaptation of typhus rickettsiae to new hosts. They were able to show that dense inclusion bodies were formed in cells of a new host although they were not demonstrable in the old host. Furthermore, continued passage in the new host was accompanied by a gradual decrease in the intensity of cellular reaction until, after many passages, dense inclusions could no longer be found.

The eosinophilic masses seen in cells of a molluscum contagiosum lesion start in the cytoplasm. As they enlarge, the nucleus is gradually displaced and compressed into a small crescent at the edge of the cell. The growing inclusion is compartmented by cytoplasmic strands or septa until it resembles multiple pockets or foci of virus (Fig. 233). At this stage, the appearance of the cell is reminiscent of multiple vesicles but there are two important differences between the foci of molluscum virus and true vesicles as seen in meningopneumonitis-infected cells. First, the small areas containing virus are not enveloped by membranes like those of vesicles. Van Rooyen [63] stated that the entire mass is enveloped by a carbohydrate-containing membrane, and he likened the inclusion to a fungus sporangium. The membrane has not been described by electron microscopists nor has its existence been denied. Secondly, except for the sporangium suggestion, there is no evidence of viral multiplication resembling binary fission or any other familiar type of biological reproduction. There is evidence, however, that the virus particles may be formed from elements of the host cytoplasm itself. In 1951, Banfield, Bunting, Strauss, and Melnick [2] showed that the strands of cytoplasm separating pockets of virus particles were honeycombed with structures representing various stages of viral development (Fig. 233). They postulated that virus was formed by the segmentation and condensation of the host cytoplasm. The first change detectable to them was the presence of a narrow band of decreased electron density outlining an almost perfect circle in the cytoplasm of sectioned cells. Its diameter was slightly larger than that of a virus particle but the material within the circle resembled cytoplasm. More advanced stages of development were represented by sharply demarcated circular holes of similar size but appearing to be empty or containing a single small dense body. The dense, internal structure varied in size but the fact that it was never larger than a mature virus particle indicated that it was probably destined to be a virus elementary body.

The study of Banfield, *et al.* was the first graphic demonstration that a virus other than psittacosis had a developmental cycle involving structures distinct from the final virus particles. In the same year, Wyckoff [67] indicated that there could be a large form of intracellular vaccinia virus. Later, Gaylord and Melnick [29] were able to demonstrate a series of developmental forms of vaccinia and ectromelia viruses resembling those of molluscum contagiosum (Fig. 234). They occurred in a juxtanuclear matrix that was dense to electrons and was not surrounded by a membrane. Similar findings have since been reported for fowl pox by Morgan, Ellison, Rose, and Moore [44], and for the virus of Shope fibroma by Bernhard, Bauer, Harel, and Oberling [10]. Apparently, developmental bodies are characteristic of the entire group of pox viruses.

The concept of *de novo* synthesis from the cytoplasm has not gone unchallenged, however. Elementary bodies of pox viruses are large and both morphologically and chemically complex. Peters, with Nasemann, Stoeckenius, and Wigand [50–54, 61], has repeatedly pointed out that there is a striking similarity between elementary bodies and some of the bacteria. All pox virus particles seem to have limiting membranes and nucleus-like zones. These structures can be demonstrated in viruses and bacteria by the same techniques and Peters *et al.* imply that the pox viruses are, in fact, bacteria. Although the study of extracellular elementary bodies has yielded valuable information regarding the structure of virus particles, there is no convincing evidence in any study of the pox group that these viruses multiply by any known mechanism. Presumably, developmental bodies do not appear intact in preparations of extracellular virus. When they are observed in sectioned material there is no sign of classical multiplication as there was in the study of meningopneumonitis. Even though it is not possible to watch living material in the electron microscope some dividing organisms, spore sacs, or ovaries should be seen in fixed material if they were present. None have ever been demonstrated. This is essentially a negative line of reasoning but the point in question is so important that analogies are insufficient as evidence. An understanding of the cytopathic results of infection and of virus multiplication depends on knowing whether the virus reproduces itself or not. Hoyle's hypothesis [35] of multiplication at the molecular level is a separate question involving only a part of a virus particle. Perhaps the pox viruses represent an organism with intermediate characteristics, or, possibly, inadequate techniques are being employed at present. Theoretically, a dividing phase could exist which is so labile that it autolyzes before any fixative can act on it.

The most recent evidence gained from studies of vaccinia [44], fowl pox [44], and Shope fibroma [10] tends to substantiate the original molluscum hypothesis regarding the origin of developmental bodies. That is to say, a limiting membrane seems to be formed directly from the host cytoplasm.[3] In addition, it is clear from these studies that the enclosed material is different from normal cytoplasm because the membranes form around patches of a granular material. In molluscum-infected cells, formation of virus is not focalized, but in vaccinia- and fibroma-infected cells a clearly demarcated zone is visible near the nucleus as an area of increased electron density (Fig. 234). It was called the "matrix area" by Gaylord and Melnick [29] and Bernhard, *et al.* [10] refer to it as a "gelatinous zone" because, as they point out, cytoplasmic streaming is arrested there at the time of infection. It is within this gel that new membranes are laid down to form developmental bodies. Subsequently, the membranes have within them all of the necessary components of a bacterial cell, and viral maturation does, indeed, resemble bacterial sporulation.

In the gelled area of an infected cell, viral developmental bodies are fairly large, delicate structures with a central, dense spot (Fig. 235). At first, it was supposed that this spot, or granule, represented a sort of embryonic virus particle [28]. The opinion of Morgan, *et al.* [44] that it is a minute organelle, resembling a nucleus, is an alternative interpretation. The granule is often surrounded by a narrow translucent band. Stoeckenius and Peters [61] have found a similar clear band in extracellular vaccinia particles. They showed, by enzyme treatment, that there is a zone of DNA-protein complex and that a clear zone results when the nucleoprotein is removed. It was also pointed out that similar clear zones are found in DNA regions of bacterial cells. It is possible that present techniques of tissue preparation for electron microscopy do not preserve DNA very well and a clear area is all that can be observed in sectioned preparations. An extensive cytoplasm-like zone surrounds the "nuclear" material but no nuclear membrane can be found. The outer membrane is quite thin.

There is a striking difference between particles seen within the matrix gel and those in the surrounding cytoplasm. Ectoplasmic and extracellular particles are smaller and more dense than those in the matrix (Figs. 234, 236). In addition, mature virus particles do not seem to have a central spot and their protein zones appear to be made up of convoluted membranes or a series of laminated membranes. Exactly what happens during the change is not clear but several things could occur. It is possible, for instance, that the first membrane to be formed arises from the host cytoplasm and that the organized viral material later forms a limiting membrane of its own within the original. In all studies of the pox viruses some forms can be seen resembling a mature particle within the primary, outer membrane. If this membrane is cellular in origin it may be that the mature virus leaves it behind or that it is in some way dissolved. It is also possible that a maturing particle tends to form folded internal membranes or threads which occupy less space than the same material would in a diffuse state. Villiform stages and more complex bar or disc forms can be observed. They usually coincide with the loss of identity of the dense granule. Whatever the process, the end result is an extracellular virus particle which is of a fixed size, dense, and remarkably resistant to environmental changes.

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[3] The objection that these membranes are dissolving instead of forming is valid but equally unprovable. Most students of the structures feel that formation is being observed, but their reasons are subjective and the original papers should be consulted.

Almost nothing is known about that part of a pox-virus life cycle taking place before the appearance of developmental bodies. It is generally assumed that an infecting particle loses its identity after it enters a cell. There is increasing evidence, however, that many, if not all, cells can ingest particulate matter at the submicroscopic level. An intact particle could enter a cell and then assume a shape not yet recognized. (Particles resembling central granules can be seen, occasionally, free in the matrix area. There is no reason for equating the two because similar particles occur in normal cells.) In such a state, the virus might well fail to survive rough handling and, as a consequence, tests for infectivity during the early stages of infection would always be negative. On the contrary, invading particles could dissolve completely, releasing infective material of some sort. It would be difficult to reconcile the ideas of the genesis of developmental bodies as discussed above if the virus particle maintained its identity.

Bang [3] studied intracellular vaccinia virus in 1950 and concluded that there was a relationship between virus particles and mitochondria. In a recent review [7] he states that although this finding has not been confirmed by subsequent investigations of others, neither has it been repudiated. Bernhard, Harel, and Oberling [10] are of the opinion that mitochondria do not take part directly in the formation of new Shope fibroma virus. Gaylord and Melnick [29] were unable to form an opinion regarding this point in their study of vaccinia. Like the question of binary fission, the concept of mitochondrial involvement is fundamental to a complete understanding of viral cytopathology but morphological evidence alone is inconclusive. In vaccinia virus infections, there does seem to be a numerical reduction in mitochondria. Furthermore, some of the villiform stages of virus developmental bodies resemble mitochondrial fragments. At times, a fragment of a mitochondrion containing a single crista is indistinguishable from some developmental bodies. It is almost futile to try to equate the two, however, because such findings are more or less rare and intermediate stages usually cannot be found. Virus within mitochondria has not been reported but large membrane-enclosed aggregates of unidentified granules occur occasionally. They correspond in size to swollen mitochondria but, again, little importance can be assigned to them because of their infrequent appearance. In other words, so many anomalies are usually seen that only repeated observations can be credited. It is quite possible that important findings are thus dismissed but there is no alternative. The very interesting intranuclear inclusions of small pox have never been studied by electron microscopy. If typical developmental bodies were found there, then mitochondria could definitely be ruled out as the site of formation.

A conscious effort to treat vaccinia, fowlpox, ectromelia, Shope fibroma, and molluscum contagiosum viruses as a homogeneous group has been made in this discussion because of their morphological similarities. Once again, however, it must be admitted that morphology alone is insufficient for final comparisons. In attempting to correlate histochemical studies with morphology, the analogies break down. Those areas of infected cells now known to contain developmental bodies have been described in the past as (a) rich in DNA for vaccinia [12], (b) lacking DNA but containing RNA for molluscum contagiosum [56], (c) containing a conjugated protein for the virus of Shope fibroma [24]. The Feulgen reactions of vaccinia-infected cells were investigated by Bland and Robinow [12] in a carefully planned time sequence study of infected tissue culture cells. It was found that inclusion bodies in newly infected cells were strongly Feulgen positive, and as they increased in size the reaction was less positive. Elementary bodies were found to be weakly positive or negative. Rake and Blank [56] studied molluscum contagiosum lesions using both the Feulgen reaction and the pyronin-methyl green technique. They found that the areas comprising pockets of elementary bodies contained DNA and that the intervening trabeculae reacted for RNA. Fisher [24] tested Shope fibroma inclusion bodies by the Feulgen technique and by RNA-ase digestion with negative results for both. The inclusions were positive for the periodic acid-Schiff technique and for protein tests. He concluded that they were composed of a conjugated protein. Thus, it would appear that three morphological analogues are chemically distinct. It may be that the differences are minor in some cases but until all three lesions are studied at the same time under identical conditions it is unfair to speculate. The vagaries of histochemical results can be controlled only by direct comparison of two samples and even then some caution is necessary. A progressive increase in the intensity of several different histochemical reactions of molluscum inclusions has been reported [41]. Such an increase might well be due to the increased mass of material being stained.

Regardless of interpretations, one fact seems to hold constant in reports on vaccinia. Early inclusion bodies seem always to be near the nucleus and basophilic. As they enlarge they tend toward eosinophila and become more variable in shape. Positive findings for DNA in small inclusions may be referable to either host or parasite and cytoplasmic gelation may or may not be caused by released nuclear material. Nuclei must have a role in cytoplasmic infection but whether it be an increase in normal function or something more direct remains to be determined. Theories of nuclear extrusion have been proposed many times but usually as one of several alternatives. Because the matrix of an inclusion body now seems to be definitely cellular in origin, and near the nucleus, the nuclear extrusion theory becomes increasingly attractive. At the same time, it hardly seems tenable if the phenomenon cannot be demonstrated in all pox viral infections. Noyes and Watson [48] found fluorescent antibody deposited occasionally in the nucleus of tissue culture cells infected with vaccinia, but the phenomenon seemed to be terminal rather than initial. In general, they found the specific antigen in the nuclear regions of the cytoplasm.

RABIES. The inclusion body associated with rabies infections is unlike those of the pox viruses because of a characteristic inner structure. The mass of this inclusion is eosinophilic but it is dotted with large and small patches of basophilic material. Negri [47] assumed that he was dealing with a protozoan when he described the inclusion body and since that time there have been varied opinions regarding its nature. The reader should refer to Van Rooyen and Rhodes for an extensive summary of the literature [63].

Contemporary opinion seems to be that Negri bodies are chiefly cellular in origin. Perhaps the best evidence in this regard is the fact that inclusion bodies are not always found even when virus is known to be present. They are not produced, as a rule, in rabbits infected with fixed virus or with certain highly virulent strains of street virus from dogs. It would appear that "fixation" of rabies virus is the selection of a virulent rabbit strain. Fixed strains have a much shorter incubation period and produce higher titers of virus than street strains. The idea that virus invasion of a new host results in formation of inclusion bodies might very well apply here.

In many respects Negri bodies do resemble Guarnieri bodies. Johnson [36] in his discussion of rabies pathology states that the inclusion bodies contain considerable lipide and no DNA. Moulton [45] has recently reported some Feulgen-positive granules in a negative ground substance but no lipides. Large inclusions were entirely negative for the Feulgen reactions. If the DNA-positive granules correspond to the classical basophilic inner bodies, then it is possible that centers of virus production occur randomly within the area of the inclusion and that they correspond to small basophilic bodies as seen in cells newly infected with vaccinia virus. Negri bodies are not confined to the perinuclear regions of neurons and the supposition that DNA may localize in them raises some intriguing questions. DNA diffusion need not occur as a free flow, however, because long extensions of the cytoplasm-nuclear interface are known to be formed in some cells and nuclear material might pass through such channels to localized areas of parasitism. (Also, as mentioned, Rake and Blank [56] demonstrated islands of DNA in all parts of molluscum contagiosum cells but the phenomenon there was generalized, no dense material being present as a limiting factor.) Alternatively, the inclusions might originate near the nucleus, as the results of Hottle, *et al.* [33] tend to indicate, and then migrate to other parts of a cell. Friedlaender [26] has found particles of *Anopheles A* virus in the endoplasmic reticulum of Ehrlich ascites cells. If other viruses are shown to be similarly located our concepts of their intracellular wanderings will have to be modified.

The bulk of a Negri body is the eosinophilic portion. Electron micrographs have shown that it is very dense [19, 33] even when fixed with formalin. It may correspond more to the gelled matrix of pox infections rather than the "plastin-like" substance but conflicting reports on the presence of lipide lead one to suspect that a three-component inclusion body is possible in rabies infections.

The virus particles themselves have not been identified as yet in sectioned material. The study of random inclusion bodies deep in the brain is a difficult task from a technical point of view and knowledge of the rabies virus will probably have to come from some other source such as tissue cultures or egg-adapted strains.

2. *Intranuclear Viruses*

Invasion of the nucleus seems to be the ultimate degree of cytoparasitism. It should be mentioned, therefore, that the spotted fever rickettsia and at least one protozoon [18] are found in the nuclei of their host cells. The phenomenon is seen more frequently in virus diseases, and recent developments have provided new concepts regarding intranuclear viral development.

Classically, the intranuclear inclusion bodies associated with viruses are described as Cowdry's types A or B, and one is left with the impression that no other possibilities exist. Polyhedral bodies of some insect virus diseases occur in the nucleus and they should be considered as a third type. More important, perhaps, is the increasing evidence that viral multiplication can occur in nuclei without the production of inclusion bodies. There is no longer any doubt that the large eosinophilic masses classified as "Cowdry A" bodies are artifacts [43, 57]. Admittedly, they are specific and reproducible but they are fixation artifacts, nevertheless. "Cowdry B" bodies appear to be real but they seem to be related only indirectly to viral development. There is, therefore, sufficient reason to believe that intranuclear viral development could proceed in the absence of any manifestation visible with a light microscope.

It is also worth while to consider the effects of a nuclear invasion relative to the cytoplasm. One would expect that damage to a nucleus might be reflected in any part of a cell but the fact that there is considerable virus traffic through the cytoplasm should not be overlooked. In addition, virus production in both nucleus and cytoplasm has long been considered possible in smallpox and more recently in herpes [57] infections.

HUMAN SKIN PAPILLOMA VIRUS. Inclusion-body papillomas were recognized as distinct lesions only recently. Strauss, Shaw, Bunting, and Melnick [62] showed in 1949 that some warts could be differentiated from the common type clinically, pathologically, and, possibly, virologically. These lesions seemed to develop more rapidly than common warts, with the production of an erythematous halo, a more pearly base, and less hyperkeratosis. In section, distinctive intranuclear inclusions and cytoplasmic masses were present. Furthermore, after maceration, a high yield of round virus-like particles could be seen in cell-free suspensions. The particles had a marked tendency to array themselves in a crystalline pattern unlike most animal viruses. The clinical and pathological features of inclusion-body warts were recognized independently by Lyell and Miles in England [40]. They later were able to confirm the presence of particles and there is little doubt that viral elementary bodies were being observed.

Although no experimental host is available, the natural lesion is ideal for the study of viral development. An orderly stratification of epithelial cells, from basal layer to the surface, represents a time sequence seldom available to cytologists. All stages of the sequence can be observed in a single section and, consequently, direct histochemical comparisons can be made. The results of a histochemical evaluation were reported by Bunting, Strauss, and Banfield [16] and a preliminary report of electron microscopic findings was published by Bunting [15]. Two unpublished electron micrographs taken by Doctor Bunting are presented here. Figure 238 is the only known electron micrograph of a type B intranuclear inclusion body. It shows conclusively that the inclusion and the virus particles are related only indirectly, a question long the subject of speculation.

Inclusion bodies are detectable in the skin papilloma in the layer of cells next to the basal layer. Intranuclear inclusions are small, round and intensely eosinophilic. Numerous small masses having similar characteristics are scattered through the cytoplasm of the same cells. As the infection progresses both types of inclusion increase in size, the nuclei enlarge and exhibit an increase in basophilic, Feulgen-positive chromatin. Electron micrographs indicate that the "chromatin" is quite probably virus. The inclusion bodies are Feulgen negative, not basophilic or subject to RNAase digestion, and they do not show selective absorption at 2650 A. In addition, they do not contain glycogen or mucopolysaccharides but they do seem to have a lipide component. At least, they show a weak affinity for sudan black B or osmium and they swell or vacuolate when the tissue is placed in dilute alcohol before fixation.

As the affected cells move higher in the Malphigian layer both types of inclusions increase in size. The cytoplasmic bodies tend to vacuolate and they become so large that they fuse to form a solid mass around the nucleus. Normal cellular differentiation is inhibited as evidenced by the failure to form intercellular bridges and the absence of typical keratohyalin granules. The cells are always larger than normal but mitotic figures are never seen.

Observations of the virus particles in sections have shown that they are packed into the nuclei in the upper layers of cells. As the numbers of particles increase they assume a linear array (Fig. 239) and finally, in the stratum corneum, they are so closely packed that a crystalline pattern is seen (Fig. 240). The pattern results from a close packing of the particles, unlike insect polyhedra where a crystal forms around the virus [42]. At this point all nuclear architecture is lost and the particles entirely fill the space which was a cell. Inclusion bodies also disappear and Bunting [15] was of the opinion that there seemed to be more virus present than could be accounted for by the nucleus alone. Figure 237 represents an early stage and it can be seen that the virus particles seem to be outside of the nucleus, in spite of an intact membrane. There are relatively few particles compared to the numbers seen in more advanced stages. One is led to the conclusion that virus developing in the nucleus is leaked into the cytoplasm continuously.

It will be remembered that the cytoplasmic inclusions appear concurrently with the nuclear inclusion in the layers immediately above the basal layer. They are numerous and scattered at random. Although virus has not been seen in association with the masses in either location, it is quite probable that the inclusion material is, in fact, obscuring the particles. It would be difficult to imagine any alternative because both inclusions keep enlarging up to the point where they disappear. When they are no longer recognizable, the virus occupies the whole cell as if it might have been present throughout all the time. Thus, it appears that the virus is physically "masked" by a cell product. Large amounts of virus can be recovered with ease but it must be derived from the "unmasked" nuclear particles.

The human papilloma virus is very small. As seen in suspensions it measures about 68 mμ when separated from other particles and about 52 mμ when packed together. After being embedded and sectioned, the size is only 28–38 mμ. Values of 73, 62, and 52 mμ were reported [36] for the virus of the Shope rabbit papilloma in suspension (depending on the method of preparation). Williams [65] has placed the figure at 45 mμ recently. Superficially, the viruses of the rabbit and human papillomas seem to have much in common. It is possible that the disappearance of the rabbit virus in a domestic host could be accounted for on the basis of cellular defenses. If unadapted virus were to multiply very slowly the cells might actually be able to lay down inclusion body material fast enough to entrap it completely. The same analogy might be used to explain the absence of demonstrable particles in ordinary human warts.

There is some indication from microincineration experiments [16] that the papilloma virus contains iron. This fact brings to mind the work of Racker and Krimsky [55] who found that the Lansing strain of poliomyelitis virus and Theiler's FA virus caused an inhibition of glycolysis in infected tissues. A specific inhibiting factor was traced to iron in the virus (Theiler's FA). The cytopathology of poliomyelitis has some features in common with that of the papilloma. Similar intranuclear inclusions occur and the normal cytoplasmic functions are upset. Howe [34] has observed that inclusions are seen only in neurons which have been attacked but not destroyed. A defensive role might well be postulated for the inclusion bodies in neurons but the analogy cannot be carried much further. The papilloma virus is probably not so small as it seems in sections (30 mμ) and to compare it with reported figures for the virus of poliomyelitis (30 mμ) would be incorrect. The latter virus was measured in suspended preparations [58].

HERPES SIMPLEX. Herpes lesions are generally characterized by eosinophilic inclusions in the nucleus. The inclusions are typical Cowdry A bodies, surrounded by a halo and resembling nothing more than a lump of shrunken protoplasm. For this reason, it has always been difficult to decide whether the inclusion body represents a mass of virus particles or cellular debris. In 1950 Crouse, *et al.* [20] showed that the inclusion, as usually seen, was the end product of a progressive change in the nucleus of an infected cell. Prior to the appearance of the eosinophilic body, the nucleus was filled with a homogeneous basophilic material which was Feulgen positive. As it increased in amount the chromatin became marginated and the nucleolus disappeared. Eventually, all of the basophilic material disappeared leaving only an acidophilic mass. This transition was found by Scott, *et al.* [59] to correspond with the appearance of infective virus outside of the cell.

A significant advance in the herpes story was made when Morgan, *et al.* [43] showed electron micrographs of infected cells containing three different structures presumed to be related to the virus. They found round "primary bodies" enmeshed in the peripheral chromatin. Primary bodies had diameters of 30–40 mμ and seemed to be related to other somewhat larger, intranuclear particles, 70–100 mμ in diameter. The latter appeared to consist of a slightly enlarged, vacuolated primary body surrounded by an additional membrane. It was postulated that the virus is formed in the nucleus and then passes into the cytoplasm. At some time during the transition a second external membrane is acquired. The particles with double outer membranes were 120–130 mμ in diameter.

Reissig and Melnick [57] followed the herpes B strain in tissue cultures and made electron microscopic examinations at known intervals after infection. Foci of developing virus were seen in the chromatin as dense condensates, then clusters of virus particles. The interesting observation that the chromatin actually seemed to be used up during virus development would suggest specific parasitism.

A reconstruction of the herpes infectious process as it appears from present knowledge follows. Herpes is a strict chromatinotrope and probably affects chromatin immediately on entering the cell. The invaded nucleus swells and, after a time, the chromatin is pushed to the margin. There is an increase in stainable DNA but it does not represent complete virus. The virus appears in small nests buried in the chromatin net at the nuclear periphery. It moves to the cytoplasm, increases in size, and leaves the cell almost as rapidly as it is produced. (During late phases, particles are scattered throughout the nucleus [57]. Centripetal spread is probably by diffusion when large members are present) There is a steady decrease in chromatin content during the infection and, at the end, the nucleus appears to be devoid of anything but a few virus particles in a thin ground substance. When the nucleus in the terminal stage is fixed with osmium no great change occurs but if it is fixed with Zenker's solution the familiar contracted Cowdry A inclusion results [57].

INFLUENZA GROUP. The viruses of influenza, mumps, Newcastle disease, and fowl plague are classed together as the Myxovirus group [1]. There is a wealth of information concerning the properties of influenza virus, in particular, but knowledge of its intracellular development is fragmentary.

With one exception [32], studies of the pathology of influenza in man or experimental animals have never revealed evidence of inclusion bodies. Special techniques designed to trace the virus in egg membranes have given somewhat conflicting results. Watson and Coons [65] found that fluorescent antibody localized in the nucleus when applied to sections of influenza-infected tissue. A photograph published by Eaton [22], using the same method, tends to show that the antigen is in the cytoplasm. Burnet [17] has discussed unpublished histochemical results of Bate indicating that the first detectable change in infected allantoic cells is a disarrangement of cytoplasmic RNA.

Early electron microscopic reports associated filamentous forms with influenza particles in suspensions and in sections of mouse lung [23]. Murphy and Bang [46] studied three strains of influenza virus in egg membranes and in tissue culture. They described uniform, dense filaments projecting from the cell surface. The filaments corresponded to those accepted as virus and differed significantly from normal "microvilli" [13]. They were unable to detect any evidence of intracellular virus but extracellular virus particles were seen as spheres and short fragments of the filaments. In addition, the average length of the filaments seemed to vary with the strain of virus and they postulated that long filaments indicated nonoptimal growth. The rapidly growing strains would, accordingly, show only short filaments (and, presumably, more spherical particles).

Evidence is available, then, to show that influenza virus in some form or other is either in the nucleus, or the cytoplasm, or on the cell border. If one attempts to correlate findings obtained from studies of related viruses the same divergent results are found. Fowl plague produces intranuclear inclusions visible by phase microscopy [25]. Mumps viral antigen is in the cytoplasm as shown with fluorescent antibody [64]. Electron micrographs of sections of cells infected with Newcastle disease give a picture similar to that just described for influenza, with important reservations.

Bang [4, 5] demonstrated clearly in his study of Newcastle disease that the observed cytopathology varied according to host cell-type on one hand and virus strain on the other. He examined three strains of virus in cultures of two cell-types and in sections of chorio-allantoic membrane. The cultured cells were derived from chick embryos and because Newcastle disease is essentially an infection of fowls, it can be assumed that mammalian host factors were eliminated. Under cultural conditions, fibroblasts were more susceptible to infection than were epithelial cells regardless of the relative virulence of one viral strain compared to another. As might be expected, virulence was strain-specific and could be correlated with the production of spherical virus particles. The highly virulent strains destroyed culture cells and virus particles were seen deep within cells of the allantoic membrane. Long projections observed with the strain of low virulence were more pleomorphic than those of influenza (Fig. 242) but, again, indicated non-optimal production of virus. No evidence of intracellular virus could be found with this strain, and, thus, it resembled influenza virus in its behavior.

If there be an underlying principle of host-virus interaction in the *Myxovirus* group it is not immediately obvious. Currently available information allows for several interpretations. Also, there is a possibility that the fowl leucosis complex being studied at Duke University [60] is caused by similar agents and carcinogenesis must be considered as a potential property of influenza-like viruses.

The most widely held opinion regarding multiplication of influenza virus is that the virus particles attach themselves to cells, penetrate, and disappear. The disappearance is thought to be a process of lysis of virus particles and concomitant release of genetic material (probably RNA). Under the influence of the viral RNA, the host cell synthesizes more virus precursor. On maturation it is liberated by extrusion from the cell in a continuous fashion to form filaments. The filaments then fragment to form spheres. This theory would almost require that the cell envelope and the virus envelope were one. That is, each virus particle would be a bit of cell wall encasing viral material. In order to account for intracellular particles as observed with virulent Newcastle disease virus some method for acquiring a membrane within the cell would have to be imagined. Current concepts of the intracellular membranes do not obviate this possibility.

Bang, in a recent review [6], observed that the virus of Newcastle disease is so pleomorphic that, except for size, it resembles the pleuropneumonia group of organisms. In the discussion of pox viruses (above) it was argued that analogies were insufficient as evidence for a similar proposal without a demonstration of cellular fission. A fission-like process is a real possibility in this case, however, because the fragmentation of filaments to form spheres resembles arthrospore formation [Fig. 243].

Discussion of intranuclear inclusions would be incomplete without the addition of pertinent recent findings.

Fawcett [23a] has studied the Lucké renal tumor of frogs and found virus-like particles resembling those of herpes. The mechanism of development appears to be different, however, and it is possible that the frog tumor agent represents still another class of intranuclear viruses. Cells of this tumor have classical, eosinophilic, Cowdry type A inclusion bodies. Fawcett showed that the inclusion material is comprised of "empty" appearing vesicles clustered in the nucleus but not associated with chromatin. Both herpes simplex and herpes B viruses were found previously, to be intimately associated with chromatin at the nuclear margin.

Other intranuclear forms of the tumor virus were seen. The now-familiar, dense central granule surrounded by a moderately dense limiting membrane was present and also, on occasion, slender filaments within the particle were noted. The filaments appeared to extend from the center to the edge of the virus particle.

Cytoplasmic and extracellular virus particles were larger than those in the nucleus and they were bounded by a double membrane. The double layer was separated by a zone of increased density giving the outer structure the appearance of a thick capsule or cortical layer.

Kleinfeld [36a] recently showed that thioacetamide-induced intranuclear inclusion bodies were actually cytoplasmic intrusions into the nucleus. These structures would ordinarily be classified as Cowdry's type B intranuclear inclusions. The connecting channel is very narrow and almost certainly invisible by light microscopy. This writer is strongly tempted to extrapolate the findings and apply them to the papilloma inclusion bodies described above. Not only were the cytoplasmic masses and nuclear inclusions of papilloma cells indistinguishable one from the other by all tests, but electron micrographs frequently show two parallel lines deep in the nucleus. As these lines can, on occasion, be traced to the nuclear membrane they may indicate a deep invagination. Although no connection between them and an inclusion body has been seen, nevertheless, it seems reasonable to suggest that Kleinfeld's discovery will be found to hold for virus inclusions. If so, the intranuclear, type B inclusion body may not be truly intranuclear.

3. Summary

In conclusion, it should be pointed out that although some beginnings have been made, progress in cytopathology can never exceed the progress being made in the study of normal cells. For instance, the bizarre forms protruding from the surface of cells infected with Newcastle disease virus (Fig. 242) are not normal microvilli. In addition, it is generally agreed that they do not resemble the artifacts caused by nonisotonic environments during fixation and dehydration. It must now be admitted, however, that they may not be unique, because Pease and Maxwell [40a] have shown somewhat similar fungating protrusions on the free surface of the epithelial cells of normal choroid plexus. It is even more important, perhaps, to realize that very little is known regarding abnormal but nonspecific ultrastructural changes resulting from cell injury, metabolic, or environmental alterations. Knowledge of the size and shape of the ever-changing mitochondria, endoplasmic reticulum, vacuoles, and other unstable components, like storage or secretory products, all needs to be worked out before it can be said that a certain observed change in fine structure is pathological.

At the other extreme are obvious pathological conditions—that is, certain tumors containing virus-like particles but without definite evidence that these particles are the causative agents. Information of this nature is being gathered rapidly now and it would be premature to attempt an evaluation of the results at present.

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16. The Dermal Ground Substance of the Mesenchyme as an Element of Natural Resistance against Infection and Cancer[1]

BY F. DURAN-REYNALS

THE SUBJECT of this chapter is a borderline region between two fields. One of them is concerned with *that function of the mesenchyme which results in changes of its ground substance:* a new field about which we are just beginning to learn something. The other field is, on the contrary, a very old one, but one about which we do not know much either: the natural resistance to infection, or, more precisely, *the nature of what is called a favorable or unfavorable ground to a given infectious agent.* The phenomena, integrating this topic, will be discussed as occurring in the skin, on the ground substance of which we have most information because of its abundance and accessibility to study.

In attempting to justify our subject we must first refresh our minds in a very general way, about the properties, extent, and distribution of the ground substance both in the embryo and in the adult. Studies already 50 or 60 years old revealed features of fundamental importance in the ground substance of the embryo [11, 47]. This ground substance was recognized as a jelly-like matter, giving the typical staining reaction of "mucins" and acting as a support for the mesenchymal cells (while the mesenchyme itself supported the rest of the tissues) and as a powerful element of cohesion; as shown by the tenacious binding of the embryonic layers [24] and by the means of locomotion that wandering cells have to use in order to penetrate it [11, 10]. Therefore, concepts were advanced defining the ground substance as a structure with very special physical and chemical features, pervading practically all tissues, and, above all, acting as a protective and binding formation. Thus *even in the embryonic stage, the value of the ground substance as a barrier was clearly suggested.*[2] Modern studies on the adult have enlarged these fundamental concepts: the ground substance of the adult is found either in a state apparently similar to that of the embryo or as a variety of matrices resulting from the progressive differentiation of this substance.

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Of a very special importance for our discussion is the abundant presence of embryonal ground substance in the skin, in a continuous layer underlying the keratinized epithelium, that is, the outer protective layer of such obvious importance in defense against infection. This arrangement already suggests an equal defensive role for the inner layer, after penetration of the epithelium by noxious agents, which is rendered more probable by the inevitable comparison of this mucinous inner dermal layer to the other mucinous layer covering and protecting the digestive and respiratory tracts.[3]

The ground substance in the adult is secreted by cells—either local or wandering, as is now much debated [4, 77]. Water and other substances pass easily from the blood into the ground substance and the component polysaccharides of the latter [66] can absorb large amounts of fluid before edema is formed. Under certain conditions, especially under hormonal stimulation, the ground substance can rapidly increase several times in volume or disappear almost entirely, and its cohesion and viscidity can undergo comparable changes. Our discussion will deal largely with whatever is known of the factors which are back of these phenomena, in other words, the endocrine conditioning of mesenchymal function.

As a means of approach to our subject let us analyze one of these effects studied by Henry Bunting, G. van Wagenen, and myself [36], the induction by estrone of that curious formation of the ground sub-

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2. It is pertinent to mention the recent work of Raynaud [76] on the development of mammary buds of the mouse embryo as being conditioned by a "mesenchymatous barrier." This perimammary mesenchyme would exert its effects "by chemical and mechanical action, probably linked to modifications of the chemical constitution of the tissue."

3. Let us point out that in studies on this digestive-respiratory mucus barrier, facts reported or reviewed in references 43, 20, 18, and 69, duplicate in several ways what will be analyzed later on the ground substance as a factor of defense. Also, the digestive-respiratory barrier can be overcome by specific actions of the invading organism, and may be a factor of vulnerability to some infections [64, 19, chapter 5].

stance known as the sex skin. In this case, the hormone, either naturally secreted or experimentally injected, causes the accumulation of large amounts of ground substance in certain regions of election in the skin of monkeys and apes. The *barrier value,* already suggested in embryonal development, is clearly shown in the sex skin, for, if one attempts to inject fluid into it by intradermal injection, one meets with considerable resistance, much more pronounced than the resistance met by intradermal injection in the ordinary skin. Another simple test on the sex skin revealing typical characteristics of the ground substance is the one consisting of pressure exerted on it with a blunt instrument; such pressure does not leave any pit, as in the clinical cases of nonpitting edema.

Resistance to both injection and pitting rapidly disappears under the effect of the agent most powerful in overcoming the barrier value, namely the spreading factor, hyaluronidase. Also, as especially shown by Bunting in the above study [36], the enzyme causes disappearance of a typical histochemical reaction, the metachromatic reaction with toluidine blue, given by some acidic, polymerized carbohydrates of the ground substance.

There are other hormonal effects resulting in a pronounced gross increase of the ground substance: the development of combs and wattles in chickens, under the effect of testosterone [56], and of exophthalmos, myxedema, and pretibial edema in humans or guinea pigs, by thyrotrophin and perhaps other hormones [57, 7, 97]. In contrast, hormones such as cortisone in rats [99, 85] and thyroxine in rabbits [3, 62, 52], etc. induce a depletion of the dermal ground substance. Further analysis of the situation would be long and confusing, for it is already known that the same hormone exerts different gross effects depending on the animal species and on still other factors. One must realize that this is a novel field no doubt most promising, but at the present moment also most obscure. The means we have to gauge the changes in the ground substance are still very imperfect, and it seems justified to state that phenomena which manifest themselves by a simple increase or decrease of the ground substance can be just as varied as the phenomena occurring in an organ in which all we can grossly detect is a state of either congestion or ischemia. Despite these limitations, however, the above facts and still others that could be given allow formulation of the following important law: *all hormonal effects carefully studied are accompanied by quantitative, qualitative, or both changes* *in the ground substance.* In some respects at least, these changes are just as conspicuous as the concomitant effects on cells, and in most cases, if not in all, these changes clearly manifest themselves in the skin.

Obviously, the ground substance of organs other than the skin also receives the hormonal impact, and this is manifested by a great variety of physiological and pharmacological actions that sometimes parallel the changes in the skin. Thus, besides the dermal effects, estrone induces formation of ground substance in the uterus [2] and decreases the permeability of the urinary bladder [80], and the other hormones, known or unknown, which—besides estrone—condition the sex cycle and pregnancy, induce a great variety of effects like changes in the rate of absorption of the synovial membrane [79] as well as in the rate of absorption from the skin [73]. Adrenal hormones, either intentionally injected or presumably liberated during stress, besides changing the dermal ground substance, suppress the permeability of the urinary bladder [80] and of the synovial membranes [79], neutralizing the effect of hyaluronidase. Whatever pituitary hormones are involved in thyroid dysfunction, responsible for pretibial edema due to accumulation of ground substance, induce the same phenomenon around the eyeballs, and in muscles as well as in the perineal, peritesticular, and axillary regions in the skin [5, 3].

From these and from still other examples that could be given, another important concept seems to be emerging, namely that of the *unity of action* of the mesenchyme, which can be expressed as follows: *when an effect is exerted on a given sector of the ground substance it is likely that the same effect will also be observed, although manifested in a different way, in some other sectors of the same ground substance.* The situation expressed in the above concept holds true despite the undeniable fact that there is, within each species, a *selectivity of response* of the ground substance from certain tissues to given endocrine effects.

Closely connected with the above is the problem of the *target cells:* How does a blood-carried hormone act on this or that cell despite the barriers—capillary wall, basement membrane, and adjacent ground substance—between the hormone and the cell? Could it be that these barriers share the biochemical make-up of the cell, and consequently combine with or react to the hormone as the cell itself would do? It has been postulated that the vascular beds share the antigenic make-up of their respective organs [75].[4] It is also known [8]—and more about it will be said later—that the skin of monkeys retains its sensitive or refractory state to estrone when transplanted into refractory or sensitive dermal regions. Then it would be far more appropriate to speak of *target tissues* rather than *target cells;* and it is amusing to consider that the ground substance, though a "barrier," by conveying to the cell the hormonal effects from the blood, would become a "pathway." In summary, both in the embryo and in the adult, the ground substance acts as a supporting and protective barrier, strongly conditioned by hormonal factors which can deplete it or build it up by a mechanism that, though more conspicuous in the skin and in target tissues or target regions of a given tissue, must be a generalized phenomenon. Thus the hormonal effects on the ground substance, though *systemic,* are of *selective* intensity.[5]

That, in man, hormonal factors are involved in the occurrence of infection and in the development of resistance against infection has been known or presumed for a long time. More recently, the intensive study of certain adrenal cortical hormones has revealed them as powerful agents, enhancing infection in most cases [86], while parallel studies have shown opposite effects by estrogenic hormones which, under certain conditions, can protect against a variety of experimental infections in animals and against natural infections in man. As reviewed elsewhere [31] and in later and exhaustive studies on constitutional [58] and induced resistance to tuberculosis [59], and also in studies on resistance to vaccinial [88] and streptococcal [44] infection dependent on hormone treatment, age [31], and still other factors, it appears that an aspect of resistance to infection resides in a change of the ground substance qualified as a *decrease in permeability,* an expression synonymous with

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[4] The view of Pressman [75] that the localization of at least the anti-kidney antibody takes place in some component of the vascular bed of the organ is based on the extremely rapid pickup of the antibody, which occurs in a single passage through the kidney: "these differences in localization must reflect differences in the vascular beds of liver, kidney, and lung. These differences in the vascular beds would be expected from the different functions of these organs which require special properties of the endothelium in transporting different substances to and from the blood." This point of view has to be kept in mind—and perhaps adapted to newer knowledge on the structure of blood capillaries—when we shall comment on the action of hyaluronidase on vascular permeability.

[5] Selye [85] and Dorfman [25a] have also speculated on the selective affinities of different tissues for hormones or other substances. In their papers they analyze facts very closely related to the subject under consideration.

an increase in the barrier value. Also, evidence was accumulated tending to show that in line with the selective, though more obvious, systemic effects of the hormone, the increase in the dermal barrier value is accompanied by a resistance generalized to the whole organism [31, 58].

Besides being directed against bacteria, viruses, and presumably other infectious agents, the resistance is also directed against cancer. A number of publications have shown conclusively that treatment of mice and rats with estrone protects them against the growth of a variety of transplantable tumors [51, 68], and treatment of human beings, with estrogenic and androgenic hormones, also has sometimes impressive beneficial effects on generalized cancer [67, 1].

The discussion that follows will center largely on the nature of the barrier value of the ground substance as a cause of resistance. Is it a purely physical obstacle which pervades the whole mesenchyme? Does its efficiency depend solely on its variable viscid consistency? Or, besides this physical value, are there in the ground substance components which, either by themselves or after undergoing changes during the complex process of inflammation [65], exert deleterious effects on invading agents? Finally, whatever the nature of the ground substance barrier, does it somehow act in concert, as a defensive element, with antibodies precisely formed in the same mesenchyme?

There are still other questions that we shall ask ourselves. However, since a given situation is much better defined if examined by comparison with exactly the opposite situation, we shall analyze the ground substance as an *element of defense,* at the same time that we examine it as an *element of vulnerability* to infection. The latter will be done by scrutinizing events attending *the intradermal route of inoculation*—that is, the situation in which an inoculum is deposited in an atmosphere of ground substance, so especially abundant in the skin. This inoculum is injected either alone or together with agents that give the spreading reaction, that is, the reaction resulting from an immediate hydrolyzing effect on the ground substance by a variety of spreading factors, the best known being hyaluronidase. We shall start with the latter situation.

The spreading factor, later termed hyaluronidase [22], was discovered by its enhancing effect on infection by intradermal inoculation [27, 31]. As an infectious agent, together with the enzyme, is injected, the inoculum spreads rapidly through the skin. The area of this initial spread is frequently the approximate area of the resultant lesions. *The barrier value of the ground substance of the skin against infection was implicitly demonstrated in these experiments, since vanquishing of this obstacle, by hydrolysis of its components and subsequent disruption of the whole structure, resulted in a pronounced increase in the size of the local lesion,* which can be followed by generalized infection. It was learned shortly afterward that many infectious agents and poisonous parasites secrete the same or comparable factors, to which they owe a great deal of their invasive power [31, 32].

When hyaluronidase is applied to tissues other than the skin it can also induce the most varied phenomena in the ground substance of these tissues. Let us mention among them: (a) the disaggregation of the granulosa cells surrounding the mammalian ovum before fertilization [31, 42, 60]; (b) absorption of materials from joint cavities and skin [40, 79]; (c) diffusion of substances through the wall of the urinary bladder [80], and (d) filtration of fluids through the fasciae of a rat muscle [25]. If hyaluronidase were applied to these tissues, in conjunction with infecting agents, one would expect that, besides the phenomena mentioned, a great variety of clinical lesions would occur of a severity presumably comparable to that of the lesions induced in the skin under comparable circumstances.

Of special relevance, in this connection, is the effect of hyaluronidase on the permeability of blood capillaries, an effect which may determine the localization in given tissues of blood-borne infectious agents either secreting hyaluronidase or injected together with it. There seems to be little doubt that the enzyme, or a factor closely linked to it, increases the permeability of capillaries [30]. The effect is exerted not on the endothelial cement, but on the perivascular sheath [103, 102], which "being a condensation of the ground substance of the connective tissue proper, presents a definite barrier to the passage of substances to and from the capillary lumen." "This perivascular sheet undergoes changes in its physico-chemical characteristics, in addition to those in the tissue ground substance, and is profoundly altered by enzymes in extracts with hyaluronidase activity" [102].[6]

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[6] This is a good opportunity to state that, as attested by what we wrote on the subject [30–32] and correcting statements by others [12a], we never concluded, first, that the active principle is hyaluronidase itself, although, despite conflicting data [12a and Elster, see 103], this may still be the case; and second, that the effect was on the intercellular cement, although we quoted [31] phrases by Zweifach, on the important role of such cement in capillary permeability, as being relevant

As said before, the pronounced increase in the size of a local lesion by spreading may be followed by generalized infection, but it might be that this generalization is not due solely to the enhancement at the outset of infection, a view that is motivated by the following considerations. Until recently, the spreading reaction was thought to be a local phenomenon. Recent contributions, however, have shown that products liberated from the ground substance, be it from its polysaccharides or from other components, exert important systemic phenomena such as the maintenance of urinary crystalloids in suspension [21] and the clearing of lipemia [82]. Further, derivatives of hyaluronic acid [33] or partly depolymerized products of it [81] obtained *in vitro* have a pronounced spreading effect and enhance infection [33, 9, 34]. Then it may well be that these are among the products liberated during the local spreading reaction, and therefore they may well contribute to the systemic effects above mentioned, let alone further dermal spreading. It may be superfluous to add that, logically, the same systemic phenomena would ensue from an attack on the ground substance at any other location besides the skin.

The above, therefore, establishes that once infection has been enhanced by overcoming locally the physical obstacle, by hydrolysis, the ground substance can become a conductor to further generalization of the infection; that is, *it changes its status from a system of defense into one of vulnerability*. Will not the host attempt to correct this dangerous situation? For, when observing the rapid spreading subsequent to the intradermal injection of a culture from a hyaluronidase-secreting bacterium, e.g., staphylococcus, one may wonder: why does not the process proceed to invade newer skin areas through secretion of more spreading factors by the bacteria located at the periphery of the lesion? In other words, how is the spreading of the infection stopped?

A partial answer to the question was given years ago when the phenomenon of the *diminishing response to successive infection* was found [35]. If virulent bacteria secreting hyaluronidase are repeatedly injected into the rabbit's skin at intervals of a few hours, even at the second injection both the spreading of the inoculum and the resulting lesion

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to the problem of the effect of intravenously injected testicular extract and comparable preparations on extravasation of dyes and other materials. We may add that work by other authors [78] does not at all allow the conclusion that the effect of such preparations is due to histamine; preparations of hyaluronidase, devoid of histamine and injected intravenously into rabbits together with Evans blue, allow rapid extravasation of the dye [34].

are already considerably reduced. A general systemic reaction has taken place that by decreasing the permeability of the ground substance—as determined by restriction of the spreading reaction—reduces the intensity of the infection. With bacteria of low virulence, not secreting hyaluronidase—for example, *E. coli*—the reaction does not occur unless the bacteria are injected in large amounts together with testicular extract or with filtrates of hyaluronidase-secreting bacteria; neither does it occur with the latter filtrates alone or with heat-killed invasive streptococcus, even if injected with the filtrates or with testicular extract. The phenomenon is not specific, for, although it is impossible to measure in the same rabbit to what extent the lesion caused by a second injection with a different bacterium has been suppressed, the spreading of, say, a filtrate of streptococcus is definitely reduced when preceded by a lesion caused by staphylococcus.[7]

It appears to be obvious that this systemic reaction is in direct relation to the danger consequent to the virulence and the invasiveness of the living infectious agent. Or, in other words, that both pathogenicity of the bacteria and hydrolysis of the ground substance are indispensable for the reaction to take place.

What are the substances responsible for this generalized effect? Are they toxins from the infectious organism alone or combined with products from the ground substance liberated during the spreading reaction? We do not know; nor do we know about the mechanism of action of whatever products are responsible for the effect. However, in view of the known systemic effect of hormones on the permeability of the ground substance, one may reasonably suspect that in this case, too, the effect on the ground substance is mediated by hormones liberated in the alarm reaction, an hypothesis which will sometime be experimentally tested. Whatever the mechanism, it is clear that, partly at least, *the defense against acute, invasive infection is due to a reaction leading to an increase in the barrier value of the ground substance.* Such reaction not only prevents the overcoming of the obstacle to infection in the areas next to the lesion, but increases much above the normal the resistance of the obstacle.

In our experiments the phenomenon has been shown to occur in the skin but must be supposed to extend to the ground substance of all tissues, a view supported by Edlund's work [40]. The intradermal injection of killed staphylococcus brings about a restriction of absorption in the knee joints of the animal and, if one knee joint is injured by a burn, the other knee joint will also show a restriction in absorption.[8]

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[7] The restriction of permeability of the dermal ground substance has also been observed by Joyner and Sabin [53] in guinea pigs chronically infected with streptococcal lymphoadenitis and tuberculosis; in the latter case there was the added interest that in the terminal, anergic phase of the disease the spread of the dye was no longer restricted.

Further useful data on which to speculate are found in the phenomenon of *critical concentration* [29]. When ten fold dilutions of a bacterium of low virulence are injected simultaneously in the rabbit skin, together with a constant amount of hyaluronidase, with the low dilutions lesions will be induced much larger than the control ones, because of the spreading effect, whereas with the high dilutions the lesion will be totally suppressed, also because of the spreading effect, which in this case has *dispersed the bacteria in the ground substance beyond the critical concentration necessary to induce lesions.* However, when the bacteria are of high virulence, like some strains of pneumococcus, no amount of spreading—that is, dispersion—will succeed in suppressing the lesion, as if a single bacterium were enough to start infection. Thus if we compare the two phenomena, (1) the suppression, *after a time interval* following the first injection of highly virulent invasive bacteria, by the diminishing response of the host, and (2) the immediate suppression by dispersion of bacteria that are much less virulent but made invasive by the added enzyme, it seems that whereas in the first instance the suppression is effected by a defensive reaction of the host, in the second case the suppression occurs in a passive host by means locally produced and immediately available. Now, it is to be remembered that whereas in the case of the *diminishing response* the suppression of infection occurs in a ground substance the barrier value of which has been increased, in the case of the suppression by dispersion the suppression occurs in a ground substance the barrier value of which has been overcome—that is, a ground substance in a state of hydrolysis. Is it, then, that in the case of the *diminishing response* the bacterium meets with a physical obstacle, built up by a systemic reaction, and that in the case of dispersion the bacterium finds itself in an unfavorable medium resulting from the process of hydrolysis? Since, however, with a bacterium of medium virulence, as with some strains of staphylococcus, the infection can be equally suppressed by both procedures, the possibility remains that, in a number of infections, both mechanisms are at play in succession, for it is conceivable that the systemic reaction to infection gets under way immediately, as infection sets in, and that therefore an immediate suppressing effect is exerted which as yet can only be effective against an infectious agent of low or even medium virulence, first made vulnerable by dispersion.

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[8] Let us point out that in Edlund's work [40] heat-killed *staphylococcus,* injected intradermally, induced the systemic reaction manifested by restriction of absorption *in the joints,* whereas, in our work, heat-killed *streptococci,* also injected in the skin, did not restrict the lesions induced by further injections *in the skin* of the same killed bacteria. Therefore, the experiments differ in several respects; they may indicate, however, that measuring of the permeability of the joint is more sensitive in revealing the systemic effects in question than measuring of the lesions from periodic injection of killed bacteria.

Whether, in the case of the *critical concentration,* the mere fact of dispersion is enough to make the multiplication of the bacterium impossible, by virtue, so to speak, of its isolation, may be considered, although the same bacterium, at the same minimal concentration, will multiply in an adequate inert medium, and fail to do so only in the live host. It is relevant, in this respect, that in general virulent bacteria that thrive so well, even if dispersed, in the ground substance are far more fastidious in artificial media than are the nonvirulent bacteria. No doubt, in both phenomena, of the *critical concentration* and *diminishing response,* phagocytosis can play an important part. Since the physical state of the ground substance is diametrically different in both phenomena, one may then wonder in which case the phagocytic function would be best exerted.

It is pertinent to mention that the phenomenon of the *critical concentration* has been invoked to explain infection by bacteria of low virulence in men during endocrine disturbance, as in the acne of puberty [88], and in mice after cortisone treatment [84]. The enhancing effect of adrenalin on the same sort of infection has also been explained on the same basis [41].

A relevant problem is whether the defense resulting from the two phenomena above analyzed holds true in the case of virus infection. The phenomenon of the *diminishing response* is not observed with vaccine virus injected in the rabbit under the same circumstances as were the bacteria [34]. This may be due, among other reasons, to the fact that in virus lesions there is no liberation of the products exerting, directly or indirectly, the systemic restricting effects observed in bacterial infection. As will be further discussed, it may also be that the rapid development of an efficient specific immunity obscures the phenomenon.

The phenomenon of the *critical concentration* with vaccinia and other viruses is not observed in the normal rabbit, infection being enhanced with extreme virus dilutions [29, 31]. Suppression of the infection by spreading, however, is observed in the partly immune rabbit [17], and this fact may well indicate a concerted action of elements of the ground substance and immune bodies. This point, too, will be further discussed.

Situations suggesting, more than do the above cases, the role of the dermal ground substance in *selective vulnerability* to infection, as well as *defense against it*—both natural and acquired—are found in plain cases of both natural and experimental infection by way of the ground substance of the skin—that is, by the *intradermal route* of inoculation. Obviously, the topic is only a facet of perhaps the most important question in infection, namely what fundamentally determines the susceptibility of a given tissue to a given infectious agent, a point so far quite obscure.

Perhaps for no better reason than that the author lived, in the Pasteur Institute, in the atmosphere where the idea developed, we shall approach the subject by reviving some points of an extremely controversial and now almost forgotten theory, that of the local immunity by Besredka [13]. We shall not at all enter into an analysis of the theory, but shall select from the effectively or allegedly supporting experiments those, probably the most sound and original, on the dermal infection by *B. anthracis*. It would appear that the skin is selectively or even exclusively —at least, with certain bacterial samples—susceptible to the bacterium. Provided the skin is not soiled, large amounts of anthrax culture can be injected by other routes, for example intravenously, with complete impunity. An elegant variant of the experiment is the following: a sealed capillary tube filled with anthrax culture is inserted in the peritoneal cavity, and a few days later, when the skin wound is healed, the tube is broken by external maneuvering, with the surprising result that no infection occurs. The bacteria have not been able to build their heavy capsules and elaborate toxic products, for, if heavily capsulated bacteria from an infected animal are inoculated by the same peritoneal route, infection and death of the injected animal will ensue [13]. Recent studies by several authors [23, 96, 87, 15] have demonstrated the sequence of events attending the intradermal infection of susceptible and naturally resistant species with spores of *B. anthracis*. In the latter circumstance the studies of Cromartie, *et al.* [23] are particularly relevant, showing that, in resistant animals, after an initial period of proliferation in the skin the bacilli lose their capsules and thoroughly disintegrate. The authors suggest "as a hypothesis that the composition of the extra-cellular fluids may change in the resistant animals as to bring about destruction of the organisms." In the susceptible species the proliferation of the spores proceeds as if they found in the dermal surrounding the components necessary for their germination and proliferation. It is in this surrounding that the bacteria will build their capsules and elaborate the toxic, aggressive products which will enable them to invade the blood stream and kill the host. But if there is interference in the equilibrium of the spore and the surroundings, as by injection of a lethal concentration of spores together with the "inflammatory factor" (that is, the factor with damaging and spreading properties secured from crude extracts of edematous dermal anthrax lesions), suppression of the infection occurs. Fully realizing the analogies with the phenomenon of the *critical concentration* brought about by hyaluronidase, Watson, *et al.* state: "the rapid dispersal of the spores caused by the damaging effect seems to enable the body to prevent the infection" [96].[9]

True, the anthrax infection seems to be an extreme case of dermal vulnerability. However, many other examples could be given of either initial infection or secondary localization as phenomena occurring selectively in the skin or rather in its ground substance. In all these cases the same question is posed as in the case of anthrax: are definite components of the dermal ground substance of a given species responsible for the implantation and subsequent proliferation of the infectious agent? In the particular case of capsulated bacteria—for example, some streptococci—could chemical analogies between the bacterial capsules and some polysaccharides of the ground substance be of special relevance to explain dermal vulnerability?

In a foregoing section, when dealing with target tissues to hormone effects, we have speculated on the possibility of the ground substance sharing the biochemical make-up of the different organs or tissues where it occurs, and for this reason—or for other reasons inherent in the ground substance itself—being physically or chemically different in different tissues or regions thereof. We also noted that blood-borne materials should theoretically go through these regional ground substances to exert their effect. If this be true, then all infectious agents—whether in the process of local primary infection by intradermal injection, insect bite or comparable means, or in the process of secondary symptomatic infection—while carried by the blood and lymph before reaching the susceptible cells should first encounter the ground substance that surrounds and presumably protects such cells.[10] Therefore, the question raised in the discussion of dermal infection by anthrax or other microbes could be repeated for the infection of many or all tissues by most infectious agents: could affinities between components of the ground substance—supposedly varying in composition according to the tissues—be one of the factors contributing to the selectivity or specificity of infection of different organs, tissues, and cells by agents circulating in the blood or lymph? This question seems to be especially pertinent in the case of agents so delicate and specific in their affinities as viruses. In parallel speculations [19, chapter 5], Burnet considers the possibility that the intestinal polysaccharide which inhibits the hemagglutination of the GDVII Theiler virus [64] serves as a point of entry for all viruses of the murine polio virus group through reversion of the virus-polysaccharide combination.

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[9] The inflammatory factor from the anthrax lesions does not contain hyaluronidase [31]. However, as shown by Julianelle, *et al.*, quoted by Watson, *et al.* [96], this enzyme is also capable, presumably by dispersion of the bacterium, of suppressing anthrax infection.

As a derivation of the above stands the significant fact—and here we pass from vulnerability to acquired immunity—that in anthrax infection the skin, the optimal organ for infection, is also optimal for immunization. The administration of Pasteur's first and second live vaccines by the intradermal route was the method first used by Besredka to immunize his experimental animals and later by other workers [see 13] to immunize sheep and other animals of economic importance. Giving precision to these observations is the recently discovered fact that the aggressive, lethal factors recovered from the anthrax dermal lesions are precisely those that are optimal to elicit efficient immune responses [96, 87].

The superiority of the intradermal route for immunization purposes to other routes—including the seemingly almost identical subcutaneous one—is not by any means limited to anthrax. A number of bacterial and viral vaccines—for example, BCG [16], influenza [94], mumps [98], poliomyelitis [89], and still others—induce an optimal immunizing response, in men or experimental animals, when inoculated intracutaneously as compared to the subcutaneous and other routes of inoculation.[11]

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[10] According to Burnet's terminology, a distinction should be made between cells in which infection is initiated, unknown in many cases, and those secondarily affected, sites of the symptomatic expression of disease [19, Chapter II].

In summary, it seems very likely, if not certain, that the events leading to the implantation of certain pathogens take place in the dermal ground substance, and it seems that the events leading to the destruction of certain pathogens also take place in the ground substance. It is fully realized that the defensive effects can be brought about by elements from the blood such as bacteriolysins, specific antibodies—let alone phagocytes—accumulated in the ground substance during phlogosis. However, these defensive elements, produced in the mesenchyme itself, may well act optimally in the ground substance and in concert with elements of it.

With the above we shall end our preliminary speculations to enter into the description of experiments aiming at results which would establish the role of the ground substance more as an element of defense than as a mechanical barrier. It is only fair to state that convincing evidence in that respect has not yet been produced. However, points of intrinsic significance have already come to light, and several of these points can perhaps be interpreted as indicating a direct inactivating effect of components of the dermal ground substance on infectious organisms and on cancer cells.

Let us first examine the *in vitro* experiments. Since hyaluronic acid is such an important component of the ground substance, and since polysaccharides and mucoid substances have been reported to exert an inactivating effect against some pathogens [90, 95, 6, 46], it was logical to study—as one of several ways of approaching the problem—the effect of one polysaccharide of the ground substance on infectious agents. Experiments in which solutions of hyaluronic acid were allowed to contact the pathogen proved inconclusive.[12] The problem was then approached

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[11] It may be instructive to quote one case where the opposite situation exists: as shown by Julianelle, heat-killed capsulated pneumococci injected intradermally into rabbits do not elicit type-specific carbohydrate antibodies, a fact attributed to effects by leucocytic enzymes [26].

[12] In these tests suspensions of vaccinia virus were incubated at different temperatures with a 1:1,000 solution of hyaluronic acid (and whatever products are

[37] by growing vaccinia virus in cultures of the Maitland type containing chick embryo cells, serum ultrafiltrate, buffer and salts, to which hyaluronic acid—from human umbilical cord and from cattle vitreous humor—was added to final concentrations of 1:1,100 or even less.[13] Thus a system more or less remotely imitating the mesenchyme was set up. The main results so far obtained in this system are the following:

(a) Vaccinia virus, passed every five days by transfer of the supernate, was inactivated in from 1 to 6 passages; hyaluronic acid from umbilical cord was more active than that from vitreous humor, and dermo vaccine was more resistant than neuro virus;

(b) The virus was protected by its association with the cell as shown by infectiousness of these cells coinciding with inactive supernates; lines of virus could easily be maintained in the presence of hyaluronic acid by passing the cells instead of the supernates;

(c) Under certain conditions lines of vaccinia virus became resistant to the polysaccharide, but in most of these cases the addition of hyaluronidase resulted in prompt inactivation;

(d) Inhibition of the virus has been obtained with glucuronic acid added to the cultures [39]. These studies, in their chemical side, have been extended by McCrea [61, 38], who has shown a direct inactivating effect on vaccinia of products of hydrolysis of hyaluronic acid by hyal-

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present after its neutralization), with the result that no inactivation occurred after one to five hours of contact. The same results were obtained when the virus was incubated with fresh extracts of tissues rich in ground substance. In other tests suspensions of *E. coli, staphylococcus albus* and *aureus,* and *streptococcus hemolyticus* were confronted with various preparations of hyaluronic acid either by incubating the bacteria and the acid or by seeding the bacteria in synthetic media containing hyaluronic acid as done by others in testing the antibacterial power of heparin [95]. Results were complex: although bacterial growth was inhibited in some tests, the results could never be reproduced with regularity. One has to consider the possibility of several factors entering here. Thus the bacteriostatic effect of heparin is inhibited by protein components of the medium [95] but enhanced by the presence of pus and blood in the region of an injury [90]. The effect of hyaluronic acid or of its split products on bacterial growth calls for much further research.

[13] Most of the hyaluronic acid was supplied by the Wyeth Institute of Applied Biochemistry. The glucuronic acid and similar compounds were supplied by Corn Products Refining Company.

uronidase, and also, in variable degree, of glucuronic acid and a series of its derivatives.

One could, perhaps, state that the results above summarized are of such nature that they can conceivably be interpreted as reflecting parallel events in the ground substance of the living animal. For the hyaluronic acid present in the cultures is not disproportionate to that which can be calculated as being present in tissues, even disregarding a possible increase in conditions of acute infection; hydrolysis of polysaccharides takes place in infection under the effect of bacterial enzymes or for other causes; and the protection of viruses and other infectious agents by their association with cells is a well-known phenomenon.

However, the results summarized have been obtained on vaccinia, precisely a virus on which there is no evidence that it is inactivated in the living wholly susceptible animal by a direct effect of components of the ground substance. Nevertheless, in the partly immune animal, vaccinial infection is suppressed by spreading beyond the *critical concentration* [17] and, since antivaccinial immunity is so quick to develop, one may well imagine a synergistic effect of immune bodies and components of the ground substance. The same could perhaps be suspected with vaccinia in the phenomenon of *diminishing response.*

One must admit, however, that even if suggestive, the phenomena on cell cultures are too far removed from reality to allow solid conclusions. Clearly, we needed an ordinary experimental animal in which large amounts of ground substance could easily be induced, in a readily accessible organ, so as to allow a much more direct type of experimentation. Because of effects of estrone in the sex skin of monkeys [36] and because the hormone was known to induce similar effects after local application on the skin of rhino and hairless mice [83], we started a study, in collaboration with Phyllis Johnson, on the effect of the hormone, injected subcutaneously, in one or several doses, into mice of a variety of strains. It was soon learned that mice of the non-inbred Swiss strain rapidly responded to the hormone—100 γ of Shering estradiol benzoate —by a pronounced thickening of the skin of the back and flanks; this reaction could easily be appraised by measuring with a caliper the thickness of a skin fold which generally reached its maximum at the fourth day. Injections of sesame oil, the solvent of the hormone, were entirely ineffective.

On such mice a number of simple tests can be carried out at once.

Thus: if 1 or 2 ml. of water or saline are injected intradermally in the flank a large bleb is formed, and from it, shortly after, a certain amount of very viscid fluid can be withdrawn with a syringe. This simple test—which also works, but only to a very limited extent, in the normal mouse—gives perhaps an indication of the physical conditions prevailing in the ground substance during some kinds of inflammation.

Another simple experiment duplicates in the estrone-treated Swiss mouse what was obtained in the sex skin of monkeys [36]: pressure on the shaved skin of the mouse flank does not leave any pit, whereas a deep and persistent depression is left when the pressure is exerted shortly after the intradermal injection of hyaluronidase. This swelling is followed at later stages by softening of a broad area around the inoculation point. The excised shaved skin of the estrone-treated mice is thick and translucent. If ground and extracted with saline, and if the resultant extracts are allowed to sediment in test tubes, the supernatant fluid appears to be viscid and rich in a fluffy material which blurs any neat differentiation of the different layers. With skin extracts of normal mice there is a clear-cut delineation between the heaviest material at the bottom and the clear supernate. As with extracts of human umbilical cord and of the sex skin of monkeys, the viscidity of the skin extracts of estrone-treated mice promptly disappears upon addition of testicular and streptococcal hyaluronidase.

The behavior of the *spreading reaction,* with and without hyaluronidase, has, of course, been studied in living and dead estrone-treated Swiss mice. It has been found that the spreading is clearly suppressed at the fourth day after injection of the hormone, coincident with maximal dermal thickening. After this time the spreading reaction reverts to normal as the effects of estrone on the skin thickness wear off. Much, however, remains to be done to relate the behavior of the reaction to changes in the ground substance—a point of intrinsic interest—and also perhaps to explain reported discrepancies.[14]

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[14] There is now an abundant and often confusing literature on the modification of the spreading reaction by hormone effects. Older data reviewed elsewhere reveal the influence, in rabbits, of treatment by hormones—for example, estrone—which exert a restricting effect on the spreading, and also of constitutional factors [32, 59, 88]. One point we want to emphasize is that, taking newer data [52] at their face value, there is no conclusive evidence that an increase in the dermal ground substance *always* induces a restriction of the reaction. For example, according to Iversen and Asboe-Hansen [52], thyrotrophin produces an accumulation of dermal ground substance and enhances the reaction, while adrenal cortical hormones deplete the ground substance and restrict the reaction [71, 70]. It is

The thickening of the skin does not begin to be clearly noticeable until 10 γ of estrone have been injected, doses of 0.5 and 1 γ being ineffective both grossly and microscopically (staining with toluidine blue). Therefore, the amount of hormone needed is much above that which is necessary to induce a positive vaginal smear [93]. As a routine, in our work, we inject 100 γ, of a 1 per cent solution of estradiol benzoate in sesame oil, subcutaneously in the groin of female mice. Under these conditions thickening is already detectable twenty-four hours later, persists for about ten days, and then disappears after another ten days. Thickening seems to persist for a longer time if the estrone is given in ten aliquots for ten or twelve days. Increase of the total body weight may also be noticeable twenty-four hours after injection.

Closer observation of the estrone-treated mice reveals the curious fact that the dermal thickening is by no means a generalized phenomenon; it is most pronounced in the flanks and back, but it is practically absent in the groins, axillae, abdomen, head, and thighs. Thus there are *marked regional differences in the response of the skin to estrone.* This phenomenon—for example, the existence in the skin of the Swiss mouse, of *target regions*—offers a splendid opportunity to study the intimate nature of the responsive or refractory states of the ground substance to hormones. Selye noticed that in his hairless and rhino mice a large cutaneous surface may acquire specific sensitivity to the follicular hormones [83]. In his transplantation experiments [8] with the sex skin of monkeys, he showed that the sensitive regions retain their hormone responsiveness after transplantation into insensitive regions, whereas the insensitive skin remains so even after transplantation into the genital region. It is needless to point out how much these facts support what was previously theorized on the autonomous properties of the mesenchyme and its ground substance, as depending on the tissues and organs in which it occurs. Developing in the mouse of the Swiss or other strains what has been started in the monkey may well throw considerable light on the problem.

These gross observations on the mouse skin are corroborated by microscopic studies carried out with Dr. R. Otis. Sections from the thick-

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obvious, however, that many factors play a part—for example, animal species or strain, length of hormone treatment, the indicator used with and without hyaluronidase, and its route of inoculation, intradermal or subcutaneous. Appraisal of all these data complemented by additional experimentation would probably show unity in the variety of results obtained by different workers.

ened flank skin show no change in the epithelia but extraordinary enlargement of the chorion in which the cells, collagen bundles, and vessels are widely separated by an amorphous material. Like the sex skin [36], this amorphous material gives a pronounced metachromatic reaction with toluidine blue, and this reaction is no longer observable after the skin has been previously inoculated with hyaluronidase as above indicated. However, the skin from the groins of estrone-treated mice is hardly distinguishable from that of the same region in normal mice.

Chemical studies on the skin of estrone-treated mice have been carried out by Dr. J. F. McCrea. The average dry weight of each skin (mostly from back and flank regions) of these mice was significantly increased—534 mg as compared to 371 mg for normal mice. The hyaluronic acid content, based on analysis of glucuronic acid and glucosamine, was increased by 84 per cent. Thus data from chemical analysis fully agree with those from gross and microscopic studies.

Differences depending on special characteristics of the strains were also found. Thus, in experiments together with Dr. R. Otis and Phyllis Johnson, mice from strains A, C3H, DBA, and C57 black, from the Jackson Memorial Laboratory, were studied, after injection of 100 γ of estrone, by measuring the skin thickness and by appraising the metachromatic reaction with toluidine blue. Results showed that mice from these strains respond to estrone far less than Swiss mice. In confirmation of Selye's work [83] however, hairless mice are as responsive as Swiss mice.

No doubt, then, genetic factors are of great importance in determining the response of the dermal ground substance to endocrine stimulus, and it seems logical to suppose that the same is true for the ground substance in general. Automatically, a study suggests itself consisting, essentially, of ascertaining the response to estrone of the whole skin of hybrids from mice of high and low responsiveness and that of transplanted skin when histocompatibility has been created. As fully justified by work to be analyzed later, it would be most instructive to ascertain the behavior of tumor cells, and also of infectious agents, in the skin under the variety of circumstances created by the projected experiments. Thus factors belonging to the regional tissue and its ground substance and systemic factors belonging to the host would be sorted out. The work of Prehn [74] should be seriously considered in this respect.[15]

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[15] Estrone-treatment of rats and hamsters did not result in any production of dermal ground substance. In work carried out together with Dr. P. Sawin, of the

An obvious point, the interrelation between estrone and other hormones, has only partly been studied. We can state, however, that cortisone, known to have a strong depleting effect on the ground substance of normal mice, entirely counteracts the effect of estrone in the Swiss mouse, so that the skin of the mice treated with both estrone and cortisone appears exactly the same as if injected with cortisone only. Work under way suggests that testosterone exerts effects similar to those of cortisone and that the effect of estrone is modified by sex, castration, and treatment with testosterone. Clearly, many other endocrine effects have to be tested, of which perhaps the most important is that of hypophysectomy. The interest of these subjects is clear; they deal with the critical problem of the proximal effect of estrone—or of other hormones equally active in this respect—on the mesenchymal cells secreting the ground substance or on the ground substance itself, a still debatable point. One has to keep in mind as a possibility of great relevance, however, that some or all known hormonal effects on the ground substance act through a common denominator.

In view of the previous discussion of the *unity of action* on the ground substance, it seems logical that estrone induces neoformation of the ground substance in tissues other than the skin. The uterus is, of course, one of the most conspicuous tissues to be considered in this regard. Significantly, in the earliest descriptions of the effect of "estrine" on this organ [2], one reads about the formation of large areas of "tissue fluid" in the stimulated uterus. Our restudy of the problem (with Dr. Otis) by histochemical methods has shown that such areas give a clear metachromatic reaction with toluidine blue. Obviously, though, much more should be done in the estrone-treated Swiss mouse—and in other strains as well—along the same lines as carried out by several investigators [101, 45]. I shall cite only one of the several inviting problems: the study of the ground substance of the central nervous system as already started by Hess [50, 49], notably in relation to the blood brain barrier. Could one venture that perhaps some light could be shed on important physiological and psychic phenomena as being conditioned by changes in the ground substance?

Jackson Memorial Laboratory, rabbits from his inbred strains IIIr, drd, Os, Ac, and Ax were treated for from one to five weeks with amounts of estrone ranging from 26 mg to 130 mg; in no case was dermal thickening induced and metachromatic matter was not in the least increased. With non-inbred New Zealand white rabbits a slight increase in thickening and in the metachromatic reaction has been obtained, but only in occasional animals.

The facts described have been so far considered for their intrinsic, physiologic value. I shall now try to relate them with phenomena of resistance to infection and cancer. What has been done on infection is not much; yet, I believe, it is already significant. Since our object is to find out whether components of the ground substance act on infectious agents in the living animal—that is, in an atmosphere of inflammation—it is logical that the first question we have to ask ourselves is whether, under the influence of estrone, ground substance accumulates in the inflamed skin as it does in normal skin. Accordingly, inflammation was induced by painting 20 times in three weeks the shaved skin of mice with a 1 per cent solution of methylcholanthrene in benzol, while other mice were painted with benzol alone. In the mice painted with methylcholanthrene the skin doubled in thickness; histological studies showed hyperplasia and infiltration of the chorion and epithelial hyperplasia, but the amount of metachromatic material was not at all increased. At this stage, 100 γ of estrone were injected in the methylcholanthrene-painted animals, and this resulted in a considerable dermal thickening, up to 6 mm skin fold. Sections of this skin gave a very strong metachromatic reaction with toluidine blue. The same phenomena, although in a lesser degree, were also observed in the mice painted only with benzol. Although it still remains to be repeated with other phlogosis-inducing agents, the above observations prove that the compounds responsible for the metachromatic reaction do accumulate in an inflamed skin under the influence of estrone.

Next, we carried out the basic experiment of ascertaining the behavior of an infectious agent, vaccinia, in the skin of the estrone-treated Swiss mice and, by way of comparison, in that of mice treated with estrone and 3 mg of cortisone and with cortisone alone, and, of course, in that of normal mice. The results were clear-cut: the mice treated with estrone responded with minimal lesions, 0.05 cm or even less in diameter, much smaller than those in normal mice; the mice treated with cortisone alone or with estrone and cortisone developed large diffuse lesions, 0.5 cm or more. Therefore, as it has been reported in the rabbit [88], vaccinial infection is very much suppressed in the skin of estrone-treated mice. Much more has to be done along these lines, the most important study being that of studying vaccinial infection, as above, in different strains of mice, trying to correlate the severity of the resultant local infection with the amount of ground substance normally present and that accumulated in the skin under the effect of estrone. Will an inverse quantitative relation be found? Or will the infection be suppressed in mice responding but little to the hormone, so that qualitative changes in the ground substance, and systemic factors as well, should be suspected as responsible for induced resistance? I must say here that vaccinia was not inactivated by contact for several hours with extracts of the dermal ground substance of estrone-treated mice, even if the skin had been previously inoculated with testicular or bacterial hyaluronidase.

Another incipient study deals with the dermal mesenchyme and its ground substance in acquired immunity. A typical experiment is as follows: estrone-treated mice were injected intradermally with vaccinia; then, at intervals of 2, 4, 8, 10, 15, 17, and 22 days pairs of mice were killed, and the virus-inactivating power of the pooled skin extracts from the region around the injected site on the one hand, and of the blood sera on the other hand, both at a 1:5 dilution, was determined in the rabbit skin. Results were as follows: inactivation of the virus was first achieved at the 8th day, and then only with the skin extracts; after the 8th day, inactivation by both skin extracts and sera was observed. So, it seems that, following intradermal infection, antibodies are first found in the dermal mesenchyme, from which they go into the blood stream. These results should be related to those on the formation of antibodies in the corneal tissue [92] and in the local lymph nodes [63], but most pertinent for our subject is the work by several authors on the same phenomenon occurring in the local skin nodules subsequent to intradermal inoculation of vaccines and other antigens [48, 91, 100]. In the estrone-treated skin of infected Swiss mice large amounts of immune material, largely devoid of cells, may be secured from which immune bodies, probably in a purer state than in ordinary tissue extracts, can be obtained, more exactly titrated, and compared to those present in the blood. Possibly this procedure would also provide a means of studying acquired immunity as superimposed on natural immunity linked with endocrine effects. I repeat that these experiments are of a very preliminary sort; but one can reasonably feel that results of significance will be obtained.

In the growth of transplantable tumors work has been carried far beyond that in the experiments so far reviewed. This part of our work was started with Dorothy McMurphy, summer student, in 1954, at the Jackson Memorial Laboratory, Bar Harbor, Maine, and is being carried out at Yale together with Barbara Stanley. The restriction of tumor growth to the site where the tumor cells are implanted, and the relatively long evolution of the resultant neoplasia, make tumors an ideal material for studying the reactions of different skin regions to invasion by foreign cells. One fact, reviewed above, has to be remembered, namely, the variable response of skin regions in the Swiss mouse to estrone—maximal in the flank, weak or nil in the groin.

Some of our experiments dealing with sarcoma 180 injected into Swiss mice four days after treatment with 100 γ of estrone, and into nontreated control mice may be summarized as follows:

(a) Five groups of mice, each group consisting of 10 estrone-treated and 10 normal animals, were injected *intradermally in the flank* with 0.2, 0.1, 0.05, 0.025, and 0.01 ml of a 1:20 suspension of tumor cells. Results were as follows: in the mice treated with estrone, tumors developed only in the mice of the first group, injected with 0.2 ml of the tumor cell suspension. These growths regressed in 9 cases out of the 10; no tumors developed in the rest of the estrone-treated mice. In contrast with that, tumors developed in 10, 7, 5, and 1 control mice injected with tumors in the respective amounts mentioned above, with the exception of the 0.01 suspension which failed to induce tumors in both the estrone-treated and control groups. In the control mice tumors regressed in 8 out of 23 animals;

(b) Two groups of mice, each group consisting of 40 estrone-treated and 40 control animals, were injected with 0.2 ml of the tumor cell suspension, the material being injected *intradermally in the flank,* in half of the animals, and *intradermally in the groin* in the other half. Results were as follows: As in the above experiment, in the estrone-treated and control mice injected *intradermally in the flank,* tumors developed in every case. These initial growths regressed in 16 estrone-treated and 7 control mice. Contrasting with that, in both estrone-treated and control mice injected *intradermally in the groin* tumors developed, much larger than those in the flank, which in both groups grew steadily until death of the host.

When in other experiments the same tumor was injected *subcutaneously* both *in the flank and in the groin,* in the flank the tumors were somewhat reduced in size with no regressions, and in the groin no differences were observed between the estrone-treated and control mice. These groin tumors were just as large as those induced in the same location by intradermal injection of tumor cells.

The study with sarcoma 180 was, to a more limited extent, repeated with the C3H-BA and the DBR-B adenocarcinomas inoculated into groups of 10 estrone-treated and 10 control mice of the corresponding inbred strains. The results may be briefly summarized as follows: both tumors, when injected *intradermally in the flank* of estrone-treated mice, grew only to about one-half the size of the control tumors with no regressions. When these tumors were injected *subcutaneously in the groin* they attained the same size as the control tumors; these groin tumors, in both treated and control mice, were much larger than the flank tumors.

This is the occasion to remember: (a) that the Brown-Pearce tumor injected intradermally in rabbits [72, 28] fared poorly and frequently regressed, whereas it grew steadily after being inoculated through other routes; and (b) that a variety of tumors which grow steadily when implanted subcutaneously may regress when injected intradermally in very small and carefully measured doses, with generalized immunity following this regression [14].

It seems logical to incriminate components of the ground substance as exerting a suppressing effect on tumor cells. These cells deposited by the injection in an atmosphere of this ground substance are rendered incapable of multiplying, and if they do, an immune reaction of the host may put an end to the initial growth.

As when dealing with the phenomena of the *critical concentration* and of the *diminishing response,* we must ask ourselves whether this effect on tumor cells is of a purely physical sort or whether a direct inactivating action on cancer cells can be suspected of occurring. One may have the impression that the viscid, rich medium of the hypertrophic ground substance—as perhaps a choice tissue culture medium—should be favorable rather than detrimental to cell growth. The work of some authors [12, 54, 55] on the enhancing or inhibiting effect of different polysaccharides on cell growth in tissue cultures or in other systems should perhaps be remembered. The possibility must also be considered that the accumulated ground substance interferes with vascularization of the incipient tumor. Some indirect light could be thrown on the problem by studying the behavior of different tumors in estrone-treated mice from inbred strains and relating the degree of inhibition to the degree of response to the hormone. Could a suppressing effect be exerted with only minimal neoformation of the ground substance, in which case qualitative changes would probably operate? Convincing evidence, however, can only come from experiments proving that extracts of the ground substance have a direct inactivating effect on tumor cells.

Whatever the mechanism of suppression of the cancer cell, the results obtained invite two further comments on the importance of the route of inoculation of noxious agents. In the first place, with cancer cells of the three tumors studied, the ground substance was *an element of resistance and never an element of vulnerability,* as was suggested in the case of some infectious agents, for example *anthrax bacillus.* Further, this resistance was far from being equally distributed in the skin, being—to judge by the available data—in direct relation to the deposition of ground substance in the chorion. This was best shown in estrone-treated mice but also, to a minor extent, in normal mice. Then, it does make a very considerable difference, in at least some strains of mice, whether the cancer cells—and conceivably infectious agents, too —are injected, not only either intradermally or subcutaneously, but also, whether by either route, in the flank or in the groin. At the practical level it is not enough, when describing a given experiment, to indicate the route of inoculation—intradermal or subcutaneous—but also the dermal region inoculated. Many contradictory results have probably been due to overlooking these points.

A last point of interest is the following: that estrone exerted an inhibiting effect on a variety of transplantable tumors in mice and rats was known for a long time [51, 68]. The described experiments seem to offer, at least in part, a coherent explanation of the phenomenon. Clinicians have reported the results, sometimes amounting to cures, obtained by treating patients with estrogenic, and also androgenic, hormones [67, 1]. In this case, it also seems legitimate to suspect effects on the ground substance as being partly behind the phenomena, and one could then speculate as to whether the quality of the results depends on the response of the mesenchyme, for individual human beings should respond as variably to estrone as strains of mice do.

The skin of estrone-treated Swiss mice seems to be most suitable, especially in relation to rheumatic disease, for study of the reactions consequent to infection and reinfection with streptococcus and other bacteria as well. This could be combined with infection by a variety of viruses to study synergistic effects between certain bacteria and viral agents. Studies along these lines have been started in collaboration with Dr. Soler-Durall.

From this discussion, it appears quite definite that the ground substance, largely controlled by hormonal and genetic factors, is the binding and supporting element of the tissues, where it is present, and the protective barrier that controls the passage of infectious agents and of whatever elements attempt to reach these tissues. This statement may appear to endow the ground substance with formidable attributes, but we have only to interfere with any of these attributes to find their existence confirmed by the significant changes resulting from their elimination, or simply their decrease, from making fertilization possible to opening the door to lethal infection. These changes, moreover, are not the arbitrary results of experimentation for, as we know, they are duplicated in the world of natural phenomena, as many virulent bacteria and poisonous animals include among their most effective weapons the ability to secrete the specific enzymes or other factors that can denature the ground substance. If the importance of a defensive entity is to be judged by the magnitude of the measures taken against it, nature is certainly pointing its finger to the ground substance, as if inviting us to learn more about it.[16]

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[16] Since the present paper was written, a number of contributions have appeared pertinent to the topics discussed. Most relevant among them are those by T. Edlund and L. Juhlin, and by L. Juhlin, published in *Acta pharmacologica et toxicologica* in 1955 and 1956. These contributions deal, on the one hand, with the effect of vasoconstriction on the permeability of the ground substance and, on the other hand, with the unresponsiveness to hyaluronidase of the ground substance of the skin previously treated with the enzyme. Both sorts of phenomena may be closely associated with that of the *diminishing response* to reinfection.

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17. *Collagen and Reticulin*

BY WILLIAM G. BANFIELD

COLLAGEN AND RETICULIN are two closely related substances which make up a major portion of the body of all vertebrates and some invertebrates. Their functions are protection, support, transmission of the energy of muscular contraction, repair following injury, and certain highly specialized functions such as the transmission of light by the cornea. They undoubtedly have other functions, such as participating in the maintenance of inorganic ion equilibrium. Fibroblasts allegedly have something to do with the production of collagen and reticulin, and the mucopolysaccharides may play an important role in the fibrillogenesis of these substances.

Collagen and reticulin have a well-defined "macromolecular" structure. Changes in the structure can be studied under a wide variety of circumstances with the aid of the electron microscope. Some forms of collagen can be put into solution and subsequently reformed from solution. Collagen is associated with cells, possesses a clear microscopic and electron microscopic structure, and maintains its "molecular" structure in solution from which its fibrils can be reformed. The following text is a review of some of the research on collagen and reticulin up to the present time.

1. Microscopic Structure

Collagen as seen in ordinary hematoxylin and eosin preparations occurs in bundles of eosinophilic fibers. Upon separation of the fibers the thinnest ones are at the limit of visibility of the light microscope. With a dark field microscope even smaller fibers are found to be present, and with an electron microscope the largest of the fibrils which make up the bundles of collagen are of the order of 2,000 A or 0.2 microns in thickness. The thickness varies depending upon the preparation and in some instances measures 100 A or less.

MATURE FIBRIL. The electron microscopic appearance of mature collagen fibrils was described independently by Schmitt, Hall, and Jakus [34] in Boston, and by Wolpers [41] in Germany. Figure 244 is an electron micrograph of a preparation of collagen shadowed with palladium. Metal deposited obliquely onto the surface of the specimen piles up on its high side, whereas it is absent or in a light film on the low side. Electrons are scattered in proportion to the thickness of the metal deposits. This increases the contrast and allows visualization of structures which previously may have been transparent to the electron beam. The shadowing is performed in a vacuum onto a dried specimen so that irregularities in contour may be the result of loss of water from more hydrated areas. This is one explanation for the areas of depression seen in a shadowed preparation of the collagen fibril. Such an explanation is open to question, since these depressions are present when collagen is prepared by the freeze-drying technique of Williams [40]. This technique is supposed to preserve the natural three-dimensional structure of the fibrils. In contrast, the depressions are absent or less prominent when the collagen is fixed in osmic acid, lead acetate, or silver nitrate, or in replicas of wet collagen.

Regardless of the nature of the contour of the native collagen fibril, the so-called "mature" fibril has a repeating transverse pattern which is duplicated on the average of every 640 A. This periodicity had previously been demonstrated by X-ray diffraction [43] and was confirmed later by direct observation [34].

Each 640 A period consists of two major bands, one dark, designated the A or D band, and one light, the B or H band [34, 41].

Upon closer observation, this repeating pattern seen in the shadowed preparation has an even finer structure. It is interrupted transversely by three or four narrower bands. The smaller bands, which also recur in a repetitive fashion, are referred to as the microperiods in contrast to the longer 640 A macroperiod. In preparations treated with phosphotungstic acid, many more bands are revealed, but their number is variable. Depending upon some as yet undetermined factors, as many as 15 bands may be found.

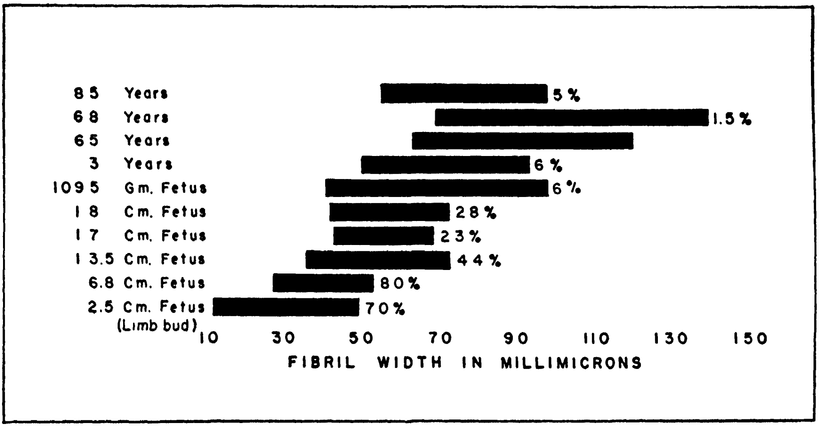
As the large bundles of collagen are composed of fibers and the fibers are composed of fibrils, so in turn, the fibrils themselves are formed of even smaller fibrils, protofibrils, which are evident as longitudinal striations (Fig. 244). Occasionally, at the frayed end of a collagen fibril, the protofibrils are clearly discernible.

The contour of the mature collagen fibril taken from a homogenized preparation which has been dried and shadowed is usually round or flattened. In some instances, however, the fibril appears as a twisted rope or composed of elements tapered at both ends. When ultra-thin sections of collagen are examined with the electron microscope after removal of the embedding media, the fibrils may appear to be tubular in structure, as demonstrated by Wyckoff [42] and by Kennedy [19] (Fig. 246). The concept of a tubular structure for collagen fibrils, however, is difficult to prove.

IMMATURE FIBRIL. For many years, all collagen was believed to possess the same structure. However, the structure of the "immature" collagen fibril differs from that of the mature fibril. Randall, *et al.* [31] demonstrated collagen fibrils with a band periodicity of 170–270 A in the tail tendon and umbilical cord of embryonic rats. Porter and Vanamee [28] found a similar periodicity in fibrils produced by fibroblasts cultured *in vitro.* This periodicity is not unique since the well-known protein fibrin has a period of 250 A [14].

When the over-all dimensions of the fibrils from a source of developing collagen are examined, it is found that the fibrils are generally narrower than those from a more mature source [4, 10, 25, 35, 36]. For example, in human skin from a 6.8 cm fetus they have a mean width of 450 A, whereas in skin from an adult the fibrils have a mean width of 1,000 A (Figs. 250, 251, Text Fig. A).

Collagen was first described as being composed of fibrils of indefinite length. The ends when seen were broken, and only rarely was a tapered, natural end encountered. However, it was subsequently learned that collagen from the tissue of young embryos or from young granulation tissue possesses many fibrils with tapered ends (Fig. 250). The number of natural ends seen will give an indication of the relative length of the collagen fibrils in a preparation [4, 2]. In some preparations, 80 per cent of the fibril ends may be tapered. This percentage is related to the age of the tissue from which the collagen originates. The age at which the number of tapered ends becomes stable for any one tissue varies. Until this age is reached, however, the percentage of fibrils with tapered ends becomes less as the tissue becomes older (Text Fig. A). This finding has been interpreted as longitudinal growth of the collagen fibril as it matures. Exactly how the growth both in length and width takes place has not as yet been ascertained. It has been suggested that "pyramidal accretion" of collagen molecules from the surrounding medium accounts for growth in length [31], while "precipitation of collagen molecules" [28] from the surrounding medium accounts for growth in width. Thus far, there is insufficient proof for either of these theories. It is altogether possible that they may represent one method by which the increased width and length results. Another possibility is that once formed, the individual fibril may not change. That is to say, the longer, wider fibrils may continue to increase by deposition anew so that the shorter narrower fibrils which were originally present become inconspicuous.



TEXT FIGURE A. Human abdominal skin. Bars represent the mean width plus and minus twice the standard deviation of the mean width of collagen fibrils from specimens of human abdominal skin of different ages. The figures to the right of the bars indicate the percentage of collagen fibrils with tapered ends in each specimen [4].

2. *Reduction of Silver*

RETICULIN VS. COLLAGEN. The coloring of the collagenous connective tissues with silver is used in differentiating reticulin from collagen. It is well known that with the silver procedures reticulin is black and collagen is brown. It has been difficult to determine whether this actually represents a real chemical difference between collagen and reticulin, or whether it reflects merely a difference in sizes of fibers, the collagen fibers being thick and the reticulin fibers thin. Collagen from the tendons of cattle [8] and reticulin from the spleen of the cat [39] prepared by Gomori's silver technique were examined with the electron microscope. The reticulin fibrils from the spleen had granular deposits of silver scattered irregularly over their surfaces. The narrow collagen fibrils of the tendon had silver deposited on them in a similar manner. However, in the thicker collagen fibrils, the silver was localized within the A bands as "molecular" deposits, thus reproducing the 640 A period of collagen (Figs. 248, 249). Although the periodic structures of reticulin and the broader tendon fibrils appear to be the same in shadowed preparations, their appearance after reducing silver is different. The pattern in which the silver is deposited does not seem to depend solely on the width of the fibril, for fibrils of the same width may be found to deposit silver on their surfaces differently [25].

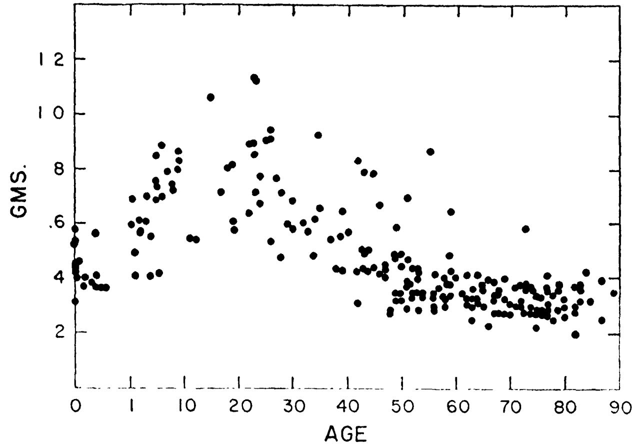
MATURE VS. IMMATURE COLLAGEN. It has been known for many years that embryonic collagenous tissue and young cultures of fibroblasts *in vitro* lay down first an argyrophilic reticulum and that this reticulum then gives way to collagen. The electron microscope reveals that the fibrils of this precollagenous reticulum reduce silver on their surfaces in the same pattern as the reticulin fibrils of the adult cat spleen —that is, the silver is deposited irregularly in large granules onto the surface of the fibrils. Electron microscopic examination of silver preparations of the collagen of tendon, bone, or sclera from man reveals that as age increases the number of fibrils taking the silver deposit in the manner of reticulin diminishes and the number taking the silver deposit in the manner of collagen increases [25, 35, 36]. Intermediate forms may be found. In some fibrils silver granules accumulate on the surface in a periodic arrangement and in others the silver is deposited both on the surface and within the fibrils. It has been proposed that this difference in the deposition of silver from fibril to fibril may be one way to distinguish young and older collagen fibrils, whereas other methods of preparation may reveal that the fibrils possess an identical appearance. It is clear that the pattern of reduced silver differs in immature and more mature collagen fibrils. However, the similarity of deposition of silver on splenic reticulin and on precollagenous reticulin is by no means final proof that these fibrils are identical—that is to say, reticulin may not always be immature collagen.

3. *Solubility and Swelling*

Two of the important properties of collagen, solubility and swelling, although not completely understood can be utilized to characterize the changing states of the collagen in the animal body.

Nageotte [23] showed that rat-tail tendon is soluble in dilute acetic acid and can be reconstituted from the acid solution by the addition of salt or by raising the pH. It was also recognized that all collagens do not have the same solubility in any given acid concentration. Whereas collagen from rat-tail tendon is soluble in a 1:25,000 dilution of acetic acid, this same concentration of acetic acid does not dissolve the collagen from the Achilles tendon of the rat. No collagen goes into solution when this concentration of acetic acid is used to extract human skin [3]. However, in a higher concentration of acetic acid (1:1,000) collagen can be obtained in solution from the skin of some human beings and not from others. In autopsy material the collagen from the skin of infants less than one year of age is generally more soluble than the skin collagen of individuals above this age. This age difference in the solubility of collagen is even more striking when human Achilles tendons are used. The collagen from the Achilles tendons of individuals over 5 years of age is not extractable with 1:1,000 dilution of acetic acid whereas all Achilles tendons from individuals less than one year of age yield soluble collagen in this concentration of acid.

The swelling of skin collagen is not amenable to accurate measurement. This is the result of a purely mechanical factor introduced by the structure of the dermis in which there is an interlacing of the collagen bundles. Human Achilles tendon, however, swells in a relatively reproducible manner when exposed to an acetic acid solution. A survey has been performed using human Achilles tendon taken at autopsy [Banfield, unpublished]. Two-tenths gram of tendon was exposed to 5 cc. of a 1:1,000 dilution of acetic acid for twenty-four hours at about 9° C. When the final weights were plotted against age, the curve shown in Text Figure B resulted. There is an increase in swelling up to the age of twenty-eight years, following which this property decreases until the age of fifty. After the age of fifty there is virtually no swelling of the tendon collagen. The gross appearance of the human Achilles tendons of different ages differs markedly after twenty-four hours exposure to the acetic acid. Those from the young individuals are turgid and translucent, whereas those from the older individuals are limp and opaque. It is possible to estimate rather closely the age of the majority of individuals from the degree of swelling and gross appearance of the Achilles tendon after it has been exposed to acetic acid.



TEXT FIGURE B. Each dot represents the weight of a 0.2 gm sample of human Achilles tendon after 24 hours in 5 ml of 1:1000 acetic acid at 9° C. The weights are plotted against the age.

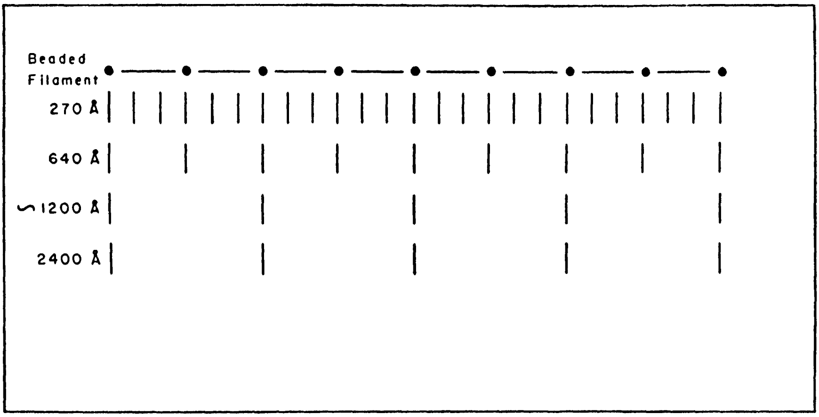
The reasons for the shape of the age-swelling curve of Achilles tendons are not immediately evident. It may be that the initial increase in the swelling properties is not the result of an increase of the swelling of the collagen *per se* but the result of mechanical factors, such as (1) a decrease in susceptibility to the action of the acid of the network of reticulin that surrounds the bundles of tendon fibers [16], (2) the binding action of the fibroblasts which are more numerous in younger tendons, and (3) a greater proportion of cellular mass to collagen mass in the young tendons than in the old. The latter part of the curve—that is, the decrease in swelling after 28 years—appears to be the result of a basic change in the collagen itself. It may be that the intermolecular bonds become progressively less susceptible to the action of the acid.

The pattern of solubility of human skin and tendon collagen in 1:1,000 acetic acid has been fairly well worked out on autopsy material.

The nature of the molecular changes in the collagen responsible for solubility changes, and the factors that initiate these changes are unknown.

There are extensive investigations in progress to find out more about the basic collagen particle. One interesting phase of this work is the study of the modifications of the structure of collagen which can be produced by varying the method of precipitating it from solution [12, 15]. When an acid solution of collagen is dialyzed against water, a gel composed of thin nonstriated fibrils is formed. If the acid solution of collagen is dialyzed against a 1 per cent solution of NaCl instead of water, fibrils possessing the 640 A periodic structure of collagen are formed. When one of the mucoproteins is first added to the acid extract of collagen in concentration of about 0.001 per cent, and the solution is dialyzed against water, typical collagen fibrils are produced. If the concentration of mucoprotein is raised to 0.05 per cent or more, fibrils are produced during dialysis, but they no longer have the 400–960 A periodic banding of mature collagen. The periodic banding is now 1,600–3,500 A. These new fibrils are called FLS (fibrous long-spacing) fibrils [12] (Fig. 245). This last experiment has at least one important implication. It shows that a mucoprotein can enter into or influence the formation of collagen fibrils. This fact has been surmised ever since the demonstration of a high mucoprotein content in regions of developing collagen. When adenosine triphosphoric acid is added to make a solution of 0.1 per cent in an acid solution of collagen, an immediate precipitate is formed of still another type of fibril, the SLS (segmental long-spacing) fibril [12, 30]. The length of the segments of this fibril are similar to the periods of the 2400 A FLS fibril, but the microperiods are different (Fig. 247). It is significant that any one of these three types of reprecipitated fibrils—the 640 A period fibril, the FLS fibril, and the SLS fibril—can be converted into any of the other two types of fibril. This interconvertibility indicates that the basic collagen particle has remained intact throughout the experimental transformations [12].

Text Figure C is a diagram copied from Randall [29] which shows the relation of the various fiber periods to one another. The embryonic beaded filaments have a 640 A period. Other fibrils in embryonic tissue have a 210 A banded period, which is about ⅓ the length of the mature collagen period of 640 A. The periodicity of the FLS fibril is twice that of the mature native collagen when the FLS fibril is formed with a large amount of mucopolysaccharide and four times the periodicity of the mature native collagen when it is formed with a small amount of mucopolysaccharide. It seems that these numbers should have some significance but it will take much more knowledge to define it. It is clear that the ability to alter the form of the reconstituted collagen fibril by varying the method of precipitation represents a unique opportunity, when combined with electron stains and chemical and physical methods of evaluation, not only to unravel the structure of collagen but also to study the chemistry of protein reactions under direct vision in a range approaching the molecular level.



TEXT FIGURE C. Relationships between spacings of various collagen fibrils. (1) beaded filament, (2) embryonic fibrils, (3) more mature fibrils, (4,5) FLS fibrils [29].

4. *Collagen Precursors and Basic Particles*

From the foregoing discussion it seems that the basic collagen unit is dissociated in the acetic acid extract and, if of sufficient size, should be directly visible with the electron microscope. Bressler, *et al.* [7], using the ultracentrifuge, computed the molecular weight of collagen to be 70,000 and the dimensions of the collagen particle to be 380 A in length and 16.7 A in width. Light-scattering methods used on acid solutions of collagen give a larger molecular weight and evidence of particle association [22]. The dimensions so far obtained are within the range of visibility of the electron microscope. Attempts have been made to visualize this basic collagen particle but none have been wholly successful. Vanamee and Porter [38] and Noda and Wyckoff [24] have published pictures of acetic acid extracts of collagen which show fibrils and filaments of varying size. They conclude, however, that the picture is not a true one but represents an artifact produced by aggregation of the particles during drying. The precursor of the mature collagen fibrils has been sought in tissue culture and in embryonic tissues. Porter and Vanamee [28] described small fibrils of 270 A period which participate in the formation of the mature 640 A period collagen. Randall, *et al.* [31] have seen beaded filaments of a 520 A periodicity in embryonic tissue, and Seki [37] describes fibrils made up of 70–80 A beads from the triceps surae tendon of a two-week-old guinea pig. It has been postulated that these filaments represent a very early stage in the formation of collagen. The work of Keech [17] is noteworthy in this respect; she studied the breakdown of collagen by collagenase and found large masses of beaded fibrils during the digestion of infant skin collagen (Fig. 253). This gives support to the theory that these beaded filaments may represent a precursor of mature collagen.

5. *Molecular Structure*

Chemical analyses of collagens show them to be made up primarily of glycine, proline, hydroxyproline, glutamic acid, and alanine. The remainder is composed of other amino acids with the exception of tryptophane, which is absent. The quantitative analyses vary in their composition sufficiently so that an attempt to construct a sequence of arrangement of the amino acids in the collagen molecule is not feasible at the present time. Several workers have studied partial hydrolysates of collagen and gelatin and have identified di- and tripeptides. Kroner, *et al.* [21] reported the isolation and identification of the tripeptide glycine-proline-hydroxyproline and the tetrapeptide glycine-(hydroxyproline, proline)-glycine. From their work and that of others, they found that a total of 68 peptides had been isolated from partial hydrolysates of collagen and gelatin, and only 20 per cent agreed with a sequence P G R[1] as proposed by Astbury [1], while 80 per cent were from some other sequence.

Spatially, the amino acids have been arranged to conform with certain physical facts. A folded grid structure [1] and various coiled structures [5, 27] have been suggested. At this writing none of these wholly fits the known facts concerning either extensibility or the X-ray diffraction data [5, 26].

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[1] P, Proline or hydroxyproline; G, Glycine; R, Some other amino acid.

Keech [17] has published electron micrographs of unit fibrils of collagen obtained by digestion with collagenase (Fig. 253). Her work suggests that a rough model of the smallest collagen unit would be a sphere or ellipse of 250 A or less in diameter with a very thin, almost invisible tail of variable length. Such units are seen separately or in chains resulting in beaded fibrils with a periodicity of 455 to 610 A. These beaded unit fibrils at times are aligned side by side, gradually fusing into a larger fibril with distinct bands. However, the specific mode of transformation or arrangement which allows these small units to form the usual, more mature collagen pattern has thus far defied analysis.

6. *Pathology*

Klemperer, *et al.* [20] were among the first to recognize a group of diseases manifested as a general disorder of the connective tissue system. These diseases had one thing in common; they were associated with a fibrinoid change in the connective tissue. Rheumatic fever, rheumatoid arthritis, lupus erythematosus disseminatus, scleroderma, scleredema adultorum, and possible allergic states such as periarteritis nodosa and thromboangiitis obliterans are considered "collagen diseases." Klemperer, *et al.* did not mean to imply that such conditions were collagen diseases *per se* rather than vascular diseases or diseases of metabolism, but that they all exhibited to a greater or lesser degree a fibrinoid change in the collagenous connective tissues. The word "fibrinoid" merely means "fibrin-like" in reference to staining qualities and does not imply a material of definite structure. The origin of this material has been a matter of debate for many years. Two theories are propounded: one is that the fibrinoid is produced by a change in the collagen itself, and the other is that it is a different material deposited around and between the collagen fibers.

Collagen is different from other proteins in that it has a high content of proline and hydroxyproline. Rheumatoid nodules containing large areas of fibrinoid were analyzed for hydroxyproline [6]. Analyses were made before and after removal of the fibrinoid. In both instances the amount of hydroxyproline present was the same and could therefore be attributed solely to the collagen content. The conclusion was that the fibrinoid in the rheumatoid nodules is not derived from collagen unless, of course, the hydroxyproline had diffused out during the formation of the fibrinoid.

Glynn and Loewi [9] found that the fibrinoid in rheumatic nodules contains a black fibrillary structure when stained with silver. This structure, like collagen, is resistant to tryptic digestion, and unlike collagen is intensely positive with the PAS reaction. Removal of polysaccharide by enzymes or by extraction results in a loss of the positive PAS reaction and of the argyrophilia. From this they conclude that the fibrinoid change is a result of infiltration of the collagen with polysaccharide-rich material.

Gueft and Laufer [13] suggest that the fibrinoid material in lupus erythematosus disseminatus is derived from nucleoprotein. This protein first is seen as hematoxylinophilic bodies within the lumen and in the walls of blood vessels throughout the body. Later it becomes an eosinophilic fibrinoid material. The nucleoprotein nature of the hematoxylinophilic bodies is established by ultraviolet absorption and by the Fuelgen reaction and the stages in the transformation of the nucleoprotein bodies to an eosinophilic fibrinoid material are traced. Rather than a collagen disease, then, they believe that lupus erythematosus may be more properly a disorder in nucleoprotein metabolism.

Rich *et al.* [32] have attempted to study altered collagen of the Arthus phenomenon with the electron microscope. They sensitized rabbits with horse serum, then injected a small amount of serum into the skin, thus producing a localized allergic reaction. In the light microscope the collagen of the skin in these regions appeared swollen and hyalinized and with ragged margins. In the electron microscope they found fibrils which had lost their striations, were broader than the original collagen fibrils, and had ragged margins. They believed that these were collagen fibrils altered by the hypersensitive reaction and that they had demonstrated a change in the collagen itself as a result of an anaphylactic phenomenon.

These changes, however, are similar to those that can be produced in collagen subjected to boiling. Figure 252 is an electron micrograph of boiled collagen fibrils which show essentially the same changes as observed by Rich *et al.* [32]. After exposure of the collagen to a temperature of 60° C for 60 minutes, similar changes were also seen but were present in fewer fibrils. One might be tempted to say that this would be expected, since breakdown of the collagen by whatever means would be likely to produce a similar picture. The destruction of collagen by collagenase, however, yields many tapered fibrils, fibrils with narrowings along their length, and small beaded fibrils [11, 17, 18]. Acetic acid causes the fibrils to swell and, on drying, to flatten out and break up into tiny filaments [33]. The way the collagen breaks up differs, at least in some instances, depending upon the method used. It seems that the experiments of Rich *et al.* [32] would bear repeating to rule out any possible alteration of the collagen during preparation, even though the altered fibrils appeared in the experimental animals and not in the controls.

7. *Summary*

The structure of the collagen molecule is not known. Though its size is in the range of visibility of the electron microscope it has not been seen. Its shape may be in the form of a sphere with a thin tail of variable length. This is suggested from the beaded fibrils seen in embryonic tissues and in the breakdown of collagen by collagenase. As seen in the electron microscope, collagen has a banded structure with the distance between bands varying depending upon the age of the fibril and other factors. The collagen fibrils are short and narrow in embryonic tissues and long and wide in most old tissues. The method by which the increase in length and width of the collagen fibrils take place, whether by accretion of molecular collagen from the surrounding medium or by initial formation of progressively larger fibrils, has not been proved. Silver is reduced as scattered granules on young fibrils and as distinct bands when reduced on most fibrils from older sources. Reticulin, though possibly not identical with collagen, reduces silver as does young collagen. Collagen can be put into solution under different conditions and reconstituted from solution by various methods to form different structures. These structures are well defined and visible in the electron microscope. This makes possible a visual study of chemical alterations of collagen approaching the "molecular" level. A greater proportion of collagen from young sources is soluble in dilute acetic acid than is soluble from old sources. In addition there is a definite correlation between the property of the collagen from an Achilles tendon to swell in dilute acetic acid and the age of the individual from which the tendon is taken. The demonstration of pathologic changes in collagen with the electron microscope has been disappointing except for the work of Rich, *et al.*, which suggests that in the Arthus phenomenon the collagen fibrils lose their striations, become broader, and develop ragged margins.

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Index

(Page numbers in italics indicate tables and text figures)

Acetabularia, 61, 64; enucleated, 63
Achilles tendon, after exposure to acetic acid, *510*
Acid-fastness, 244–5
Acid hematein test, 311, 323
Acidophil cytoplasm, 290
Acidophiles, 274 f.
ACTH. *See* Adrenocorticotropin
Actin, 362–4
Actomyosin, 401
Adenine, 103, 122, 175 ff., 206–7; "active," 210; 2-substituted, 87–90
Adenohypophysis, 322, 331
Adenosine triphosphatase (ATPase), 428
Adenosine triphosphate (ATP), 87
Adipose tissue, 245–8
Adrenal glands, electron microscopy, 334
Adrenalectomy, 273
Adrenocorticotropin (ACTH), 275, 278
Agranular reticulum, 315–18, 322, 324–5, 328–9, 332
Alanine, 207
Alcohol-trichloracetic acid, 271, 274
Aldehyde reaction, 244
Amino acids, 89
Amitosis, 396
AMP, 210–11
Anaphase, 103–4; sticky bridges in, 103–4
Anoxia, 15
Antagonism tests, 92 f.
Anthracis bacillus, 483–4, 497
Anthrax infection, dermal vulnerability, 484
Antibiotics, 47
Antimetabolite hypothesis, 103
Antitemplates: in differentiation, 409 f.; in regeneration, 409 f.
Apocrine glands, 320; cytology of secretion in, 325–8
Arginine, 139
Ascaris, 38
Ascites cells, nucleic acid content, *216*
ATP. *See* Adenosine triphosphate
ATPase. *See* Adenosine triphosphatase
Autosynthesis, 403
Azaserine, 104 f., *105*

Bacillus anthracis, 483–4, 497
Bacteria, viruses, 181
Baker's acid hematein, 311, 323
Baker's sudan black method, 310–13, 314 f.
Barrier value: of ground substance of skin, 478, 481; increase in, 477, 480
Basophiles, 275
Basophilia, 9, 182, 244, 286–91, 321, 323–4
Beer-Lambert laws, 116 f.
Birefringence, 20, 43, 48, 59, 358–9; of cilia, 24; negative, 59
Blastocyst, 14
Border
  brush, 22–4
  striated, 19–22, 24; pore vs. rod controversy, 20
Bridge-break-fusion cycle, 104
Brush border, 22–4

Caffeine, 105; 8-ethoxycaffeine, 106
Canaliculi, 12, 23
Cancer, resistance against, 472–98
Carbohydrates, 9
Cells
  absorptive function, 11
  acinar, 328–31
  adjacent, *34;* specializations of contact surfaces of, 29–36
  ascites, nucleic acid content of, *216*
  base of, specialization of, 36–8
  blastocyst, 14
  boundaries, 35 f.
  cohesion, 30
  cycle
    desoxyribonucleoprotein - complex, 113–55

Cells (*continued*)
        stages (*see also below,* interphase stages): autosynthetic interphase, 114; heterosynthetic stage, 114; mitosis, *114*
    differentiation, 381–411
    endothelial, 11
    enucleated, 63–4
    enzymes, cellular, 10
    free cells in vitro, 388–90
    free cells in vivo, 386–8, 389
    glial, 12, 16
    goblet, 325–8
    histiocytic, 389; macrophagic, 390 f.
    interphase stages, 114, 147
    isolated, occurring in regenerating lesions of muscle, 386–94
    lipide inclusions within, 15, 230–1, 235–9
    macrophagic, 390–4; causative factors in transformation, 392; fate of, 391–2; other forms of isolated free cells, 392–4; properties of, 390–1
    microvilli found on mucous neck, 21
    moribund, 14 f.
    nucleus, 42–55, 113–55
    pancreatic, 13, 287, 328–31
    parenchymatous, 16
    pathological swelling, 417–39
    reaction during virus infections, 447–68
    reverting postmitotic, 381
    Schwann, 12
    sebaceous, 323
    steroid-producing, 243
    structure, cellular, 9–17
    surface: interdigitation of confronted, 33; lateral, 36; structural specialization, 19–38
    swelling of, 429–30
    target, 475
    tissue culture, 84–112, 433–8
    transformation, 381–411
    turgid, 15
    vacuolization, 417–39
    virus infections, reaction during, 447–68
Cement, intercellular, 29–30
Cholesterol esters, 241–3, *242*, 251; Schultz test, 251–3, 254
Chromatin, 46, 52; filaments, *see* Chromonemata; PNA in, 191
Chromidial substance. *See* Ergastoplasm
Chromonemata: helical coiling, 56; multiple, 56
Chromophore, 116
Chromosomal damage, 99–104
Chromosomal proteins, 137–50
Chromosomes, 42–66, 150–4
    breakage, 104–7
    chemical aspects, 59–60, 142–5
        removal of DNA, 143; enzymatic, 143; salt extraction, 143
    constitution of, 55–9, 142–5
    damage, 99
    DNA removal, 143
    giant, 58–9
    isolated, 56–8
    polytene dipteran, 151–2
    salt-isolated, 45
Chrysoidin Y, 315
Cilia, 24–7; intracellular portion of, 25; birefringence, 24; rootlets, 26; structure of, 25, *28*
Cloudy swelling, 417–33; protein changes, 430–1
Coenzyme A, 95, *96, 97, 100, 101*
Colchicine, 47, 135; inhibition of mitosis, *136;* treatment, 133
Collagen, 26, 504–17
    electron microscopy, 515
    fibrils, *512*
    immature fibril precipitation of, 507
    mature vs. immature, 505
    microscopic structure, 504
        immature fibril, 506; "pyramidal secretion," 507
        mature fibril, 505–6; repeating transverse pattern, 505
    molecular structure, 513–14
    pathology, 514–15
        high content of proline and hydroxyproline, 514–15
    precursors and basic particles, 512–13
    solubility and swelling, 509–12
Concentration, critical. *See* Critical concentration
Contractile substance, 353–4
Contractile vacuole. *See* Vacuoles
Contractility, 343
Corpuscles: basal, 24; macrophage-like, birth-process of, 389
Cortisone, 273, 492 f.
Cowdry's types: A bodies, 460, 463, 467; B bodies, 460, 467
Cristae mitochondriales, 14
Critical concentration, 484, 488, 496; of bacterium, 481, 482; with vaccinia, 482–3
Cross striation, 399–400
Cyanide, potassium, 269
Cytochondria, 366–7, 424–5

Cytodifferentiation, 382, 402–3; current concepts of, 404–407; sarcoblasts, 404–7
Cytogenetics, 42
Cytophotometry, 114, 134; errors in, *118–19;* physical basis of technique, 115
Cytoplasm, 283–301
  acidophil, 290
  differentiation, 402
  functions, 167–219
  structure, 320–1
  tissue culture cells, 435–6
  viruses, 448–60; pox group, 451–8; psittacosis group, 448–51; rabies, 458–60
Cytosine, 122, 175 ff.

DDD, 269–72
De-differentiation, 382
Degeneration: dissociative, 387; hytropic, 423–4; parenchymatous, 418–33; vacuolar, 423–4
Degenerative granules, 420
Dermal ground substance of mesenchyme, 472–98
Desmosomes, 29, 30–1, 33
Desoxyribonucleic acid (DNA), 53–5, 59–60, 85–6, 120–2, 153, 422, 455, 457, 459, 464
  analysis, *202*
  autosynthetic interphase, 126–7
  biological properties, 121
  changes during cell cycle, 113–55
  constancy, 122–4
  enzymatic depolymerization of, 130
  frequency distribution of amounts, *125*
  and histone, 139–40
  nuclear size, *128*
  polynucleotide chains in, *175*
  synthesis of, 125–7, *130;* DNA, rate of, *132;* mechanism, 128–34; and mitosis, 86, 134–7; relationship between synthesis and cell division, 134–7
Desoxyribonucleohistone complex, 145
Desoxyribonucleoprotamine, 144
Desoxyribonucleoprotein, 120
  complex: behavior of, 148, *148,* 150; changes during cell cycle, 113–66
Diaminopurine, 103
Diazo blue B, 267
Diazonium salts, 266–8, 269
Diestrus, 35
Differentiation, 397; antitemplates in, 409 f.; cytoplasmic, 402; nuclear, 401–2
Digitonin, 254
Diminishing response, 482, 488, 496; with vaccine virus, 482
Disulfides, 269–72
Division, indirect. *See* Mitosis
Drosophila, 43

Ecology, molecular, 405
Ectromelia virus, 452
Edestine, 285
Electron microscopy, 10, 21, 23, 26 f., 30 f., 36, 43, 47, 283–304, 314 f.
  adenohypophysis, 331–2
  adrenal gland, 334
  brush border, 22–4
  cell surface, 11, 29–38
  cilia, 24–7
  collagen, 515
  cytoplasmic structure, 320–1
  desmosomes, 30–1
  epithelia, 32
  flagella, 27–9
  goblet cells, 326–7
  Golgi complex, 314–18
  intercellular bridges, 30–1
  intercellular cement, 29
  microsomes, 283–91, 293–301
  mitochondria, 14–16
  myofilaments, 361
  pancreas, 13, 328–31
  placenta, 13
  plasma membranes, 11, 19–38
  sarcolemma, 351–2
  sarcoplasmic reticulum, 371
  sarcosomes, 369
  sebaceous cells, 324–5
  secretory granules, 322
  skin, 30
  striated border, 19–22
  striated muscle, 359, 361–4
  terminal bars, 31
  thyroid, 13–14, 332–4
  viral inclusions, 448–67
Elliptio, 21
Embryo skin cultures, *102*
Endocrine glands, cytology of secretion in, 331
Endoplasmic reticulum, 11, 13–14, 287–90, 292–8, 315–18, 324–5, 335–6
Enzymes, 9; cellular, 10; development of, *198*
Epidermal mitosis, 86

Epididymis, 308 f., 314 f., 319
Epithelia, 11, 20 f., 31, 37, 289–90; electron micrographs of, 32; renal, 23; squamous, 35; stratified squamous, 32
Ergastoplasm, 11, 13–14, 286–7, 307, 324, 326 f., 330 ff.
Esters, cholesterol, 241–3, 251
Estradiol, 214
Estrone, 475, 490 f., 493, 495, 497; interrelation with other hormones, 492; response of skin to, 490
Estrus, 35
Ethoxycaffeine, 105

Fast blue RR, 267
Feulgen reaction, 120–1, 129, 147; and methyl green staining, 131
Fibrillation, 399, 402
Fibrils, 398–9
  collagen, *512;* FLS (fibrous long-spacing) 511; SLS (segmental long-spacing) 511
  sarcoblasts, 398–9
Fibroblasts, proliferating, 13 f.
Filaments: actin, 362–4; in cilia, 24; in flagella, 27 f.; myosin, 362–4
Fixation, 16; effect of, 231–6, *231;* by osmium tetroxide, 43
Flagella, 27–9
Fluorescence, 244
Follicular cells, thyroid, 11, 332–4
Formalin, 231–6, 268, 270
Fractions: microsomal, 295–8; post-microsomal, 298
Free surface, specialization, 19–29

Galactolipide, 311
Genetic equivalence, 406
Glands: adrenal, 334; apocrine, 320, 325–8; endocrine, 331; holocrine, 320; merocrine, 320; steroid-producing, 251–3; thyroid, 13, 332–4
Glial cells, 12, 16
Glutamic acid, *197*
Glutathione, *95,* 267
Glycogen, 368
Glycosuria, 273
Goblet cell, 325–8; distribution of radioactivity in, 327
Golgi complex, 307, 308–34
  identity of, 308; as artifact, 309 f., 312; synthetic theory, 310; ultrastructure of, 316
  secretion and, 320–34
Granules: in cloudy swelling, 418–19; degenerative, 420; resorption, 420; secretory, 322, 420; starvation, 420; zymogen, 13, 329 f.
Ground substance: binding element of tissues, 498; element of defense, 486; unity of action, 492
Guanine, 89 f., 93, 122, 175 ff., 206–7
Guarnieri bodies, 452, 459

Habrobracon eggs, x-irradiated, 135
Helix, 121, 174, *175;* cortical, 27
Hematein, 311, 323
Hemoglobin, 188
Hepatic parenchyma. *See* Liver
Herpes simplex, 463–4
Heterosynthesis, 114, 403
Histiocytic cells, 389; macrophagic, 390 f.
Histochemistry: protein, 264–82; specificity vs. localization, 9–10
Histolipides, 228, 244
Histones, 138–41, 145; and DNA, 139–40; removal of, 147; staining, 120, 148
Holocrine glands, 320; cytology of secretion in, 322–5
Hormones, 212–15, 474
  estrone, 492
  protein, 264–79; demonstration of, 272–8
Hyaluronic acid, 486
Hyaluronidase, 474 f., 477 f., 479–80, 481, 484, 489
Hydrogen-bonding, *173*
Hydropic degeneration, 423–4
Hydroxyproline, 514–15
Hypothesis, antimetabolite, 103
Hypoxanthine, 89, 93

Inclusion bodies: cytoplasmic, 448–60; nuclear, 460–7
Infection: and cancer, resistance to, 493–4; defense against, 483; resistance against, 472–98; selective vulnerability to, 483; virus, 447–68
Influenza group, 464
Inoculation, intradermal route, 483
Insulin, *98,* 100, *100,* 273

Intercellular: bridges, 31; cement, 29–30
Interphase, 44, 114; autosynthetic, 114, 126–7
Intestine, striated border of, 22
Intracellular: membranes, 13, 287–90, 292–8, 315–18, 321, 328–30, 332–6; lipides, 227–54; virus, 460–8
Ischaemia, 393
Isolation (of cell constituents), 292–3, 295–301
Isotope labeling studies, metabolic activities of PNA's, 204–10

Karyokinesis. *See* Mitosis
Karyolymph, 50
Karyotheca. *See* Nuclear membrane
Kidney: mammalian, 35; proximal convoluted tubule, 38

Lipides, 9, 54–5, 58, 227–63
  acidic, Nile blue sulfate straining, 254
  adrenal, *252*
  brown-fat, 248
  constant, 228
  droplets
    significance, 245–54
      adipose tissues, 245–8; hepatic parenchyma, 248–50; steroid-producing glands, 251–4
    histochemistry, 245–54
  functional, 228
  inclusion, 15, 368
  intracellular, 227–54
  metabolic, 228
  methods of demonstrating, histochemically, 230
  phosphorus, growth ratios, *200*
  pigments, 243–5; reactions, 244
  pseudoplasmal, 233
  reserve, 228
  state of, in biological structures, 227–30
  structural, 228
  unsaturated, 254
  variable, 228
  white-fat, 246–7
Lipoic acid, 96–7, *97, 100, 101,* 106
Lipochondria, 312 f., 318 f.
Lipophanerosis, 228–30
Lipoprotein complexes, 228
Liver: biochemical analysis of, *194;* microsomes, 296, *297;* parenchyma, 248–50
LTH, 278
Lumbricus, 21
Lysine, 139

Macromolecules, 283–304
Macrophage, 393, causative factors in transformation to, 392
Macrophagic cells. *See* Cells, macrophagic
Malpighian tubules, of insects, 37
Maturation, 397, 400–1; increase of proteins, 401; re-differentiation, 400–1
Membrane: agranular, 324–5; intracellular, 13, 287–90, 292–8, 315–18, 321, 328–30, 332–6; nuclear, 48, 50; plasma, *see* Plasma membranes
Mercaptopurine, 90, *91,* 92, *95, 97, 98, 99, 100, 101,* 104
Merocrine glands, 320; cytology of secretion in, 328
Mesenchyme, 472, 477; dermal ground substance of, 472–98; unity of action, 475
Metaplasia, 383; regressive, 394
Methyl green staining, *126,* 129, *146,* 147–8
Methyl purine, 91
Methylene blue, 437
Microdissection, 49, 64–5
Microlabyrinths, 13
Microscopy, electron. *See* Electron microscopy
Microscopy, ultraviolet, 9
Microsomal fractions, 295–8; isolation of small particles, 299–301; morphological and biochemical analysis, 295–8, *297*
Microvilli, 11, 13, 20 ff., 35, 37
Mitochondria, 10 f., 14–16, 20, 37, 52, 95 f., 244, 246, 307, 314, 321, 323–4, 326, 329, 332, 334, 365 ff., 397 f., 421 f., 456–7
  cloudy swelling examination of, 422–4
  crescent formation, 427 f.
  cristae mitochondriales, 14
  filamentous, 62
  heart-muscle, 426
  hypotonic solutions, 428
  of muscle, 368–9
  nonosmotic transformation, 429
  swollen, 16
  in tissue culture cells, 433–4
  in vitro, 427
Mitosis, 43 f., 46, 64–5, 84–107, *114,* 133, 149 f.; anaphase, 103–4; colchi-

Mitosis (*continued*)
  cine inhibition of, *136;* and DNA synthesis, 86, 134–5; epidermal, 86
Mitotic incidence, 92–9
  purine, *89, 94;* unsubstituted, *88*
Mitotic poisons, 87–8
Molecular ecology, 405
Mollusc: elliptio, 38; structure of ciliary apparatus, *25*
Mononucleotides, soluble, occurrence in tissue extracts, 185–6
Morphogenesis, 186–9
Mouse sarcoma, *102,* 180
Mucins, 326 f., 472
Mucopolysacchride, 511 f.
Muscle
  contraction, 343–5
  isolated free-cells of muscular origin, 392–5
  mammalian striped, 383–6
  protoplasm, plasticity of, 407
  striated, 346–8; regeneration, 381–411
  striped: cytology of, 343–73; distribution of, in animal kingdom, 345–6; mammalian, 383–6; myofibrils, *see* Myofibrils; sarcomere, 362; sarcosomes, 366–9
Mustards, 104; nitrogen, 104; sulfur, 104
Mutagenicity, 104–7
Myoblasts, 393
Myofibrils, 353–64, 398, 399–401; separation from sarcolemma, 353
Myofilaments, 361–2
Myosin, 344, 362–4
Myotendinal junction, 372–3

Naphthol, 267
Negri bodies, 458–9
Neoplasia and PNA metabolism, 215–17
Neoplastic tissue, 88
Nephron, brush border, 22
Neurilemma (Schwann cells), 12
Newcastle disease, 465 f., 468
Nile blue sulfate, staining, 254, 315, 436
N-isomaleimide, 268
Nissl bodies, 288
Nitrogen mustards, 104
N-maleimides, 268
Nonhistone, 141–2
Nonosmotic swelling, 428
Nucleal reaction. *See* Feulgen reaction
Nuclear differentiation, 401–2; sarcoblasts, 315–16, 401–2
Nuclear function, 167–218
Nuclear membrane, 48, 50
Nuclear sap, 50
Nuclei, 42
  anisotropy of, 44
  chemical composition of, 52–5
  fixed, 46
  flattened, 116
  functions, 60–6; heredity, 61; interactions of, 61–3; reproduction, 61
  interphase, 44
  membrane of, 43–4
  plant, 44
  proteins of, 53; histone, 53; protamine, 53
  types of: bacteria, 46–7; oocytes, 46–7
Nucleohistone, 143
Nucleoli, 307; chemistry, 60; multiple, 47
Nucleoproteins: biological role, 150–4; desoxypentose, 9, 137; during cell cycle, 145; pentose, 9, 179–80
Nucleotides, 170–2, 174–9, *190;* composition of pentose nucleic acids, *176*

Oocytes, 47; morphological studies of, 50
Osmiophilia, 366 f., 449
Osmium tetroxide, 43, 236, 284 f., 295 f., 299, 307 f., 310, 312
Osmoregulation, 425 f.
Osmosis, 426
Osmotic swelling, 426

Pancreas: electron microscopy, 13, 286, 290, 328–31; metabolic potentialities of PNA, 202; microsomes, 295; particulate component in, 287
Papillomas
  human, 462; viruses of, 460–3
  inclusion-body, 460
  skin, 5
Paramecia, 12
Parenchyma, hepatic. *See* Liver
Particulate component of cytoplasm, 283–301; chemical nature, 291; distribution, 285–91; morphology, 283–5; relations with endoplasmic reticulum, 293–5; relations with microsomes, 292–3
Pentosenucleic acids (PNA), 167–226, 397 f.
  analysis, *202*
  biosynthesis of, 209–12
  cellular distribution of, 181–5, 186–212

    concentration of cytoplasmic RNA, *192*
    cytochemical studies, 186–95, *188, 189*
  chemistry of, 170–4
  isolation of, 168–70
  metabolic activities, 203–10
    chemical studies of PNA metabolism, 195–203
    extrinsic effects, 212–15
      hormonal factors, 212–15; neoplasia, 214–15
  microsomes, 184
  nuclear, 208
  nucleotide composition, *176*
  purines and pyrimidines, *178*
  types, 172; nucleotide and base components of, 174–9
Pepsin, 143
Periodic acid-Schiff reaction, 23, 276–7, 326, 390
Permeability, decrease in, 476
Peroxide reaction, given by lipide pigment, 244
Phosphatides, 239–41, 254; destruction of, 232–3; staining of, 239–40
Phospholipide, 311, 315, 323
Phosphorus "turnover" during DNA synthesis, 133
Pigmentation, 311, 315, 323
Pilocarpine, 328 f., 331
Plant viruses, 181
Plasma membranes, 11–13, 353
Plasmalogens, 235, 241
Plastin, 449 f., 452
PNA. *See* Pentosenucleic acid
PNA-N, 202–10
PNA-P, 205–10, 213
Poisons: mitotic, 87–8; radiometric, 87
Poliomyelitis, 463; infected tissue cultures, 426–7
Polymerization of DNA, 129
Polynucleotide, chains in DNA, *175*
Polyploidy, 123, 135, 214; changes in frequency, *136*
Polysaccharides, 486; hydrolysis of, 488
Polyteny, 123
Postmetaphases, *102*
Postmicrosomal fractions, 298
Potassium cyanide, 269
Pox group, 451–8; pox-virus life cycle, 456
Prefibril stage, 398
Proline, 514–15
Protamine, 138–9, 144 f.
Proteins
  chromosomal, 137–50
  histochemistry, 264–79
  hormones, 264–79
  maturation, 401
  nature of, in PNA-protein complex, 179–81
  nitrogen, growth ratios, *200*
  and nucleic acids, *196*
  nucleoproteins: desoxypentose, 9, 137; pentose, 9, 179–81
  residual, 141–2, 145, 148
  sulfur-rich proteins, 9
  synthesis, 152–4; cytoplasmic, 152; DNA in, 152
Protofibrils, 399
Psittacosis group, 448–51
  cellular infection, 450
  inclusion body: parasite, 448 f., plastin-like substance, 449 f.
Purines, 104, 122 f., *178*
  6-chloropurine, 90
  chromosomal damage, 101–4
  effect on mitotic incidence, *95*
  6-mercaptopurine, 90, *91*, 92, 95
  methyl, 91
  6-methylpurine, 91
  6-substituted, 90–9, *90, 91, 92, 93*
  unsubstituted, 87–90, 92–3
Pycnosis, 150
Pyrimidines, 122 f., *178*
Pyronine, 315

Rabies, 458–60
Radiomimetic poisons, 87
Re-differentiation of striated muscle, 382, 396–408; cross striation, 399–400; fibril stage, 398–9; maturation, 400–1; prefibril stage, 397 f.
Refringence, 366
Regeneration, 394, 408–11; antitemplates, 409 f.; fixed postmitotic cells, 381; physiological, 381; injury, 385–6; ischaemic infarct of muscle, 384–5; skeletal muscle in vitro, 386
Regressive metaplasia, 394
Resistance, ground substance an element of, 497
Resorption granules, 420
Reticulin vs. collagen, 504–17
Reticulum: agranular, 315, 317, 326; sarcoplasmic, 369–72. *See also* Endoplasmic reticulum
Rhabdomyoblasts, 408
"Rhombosomes," 398
Ribonucleoprotein, 299 f.

RNA, 53–4, 60, 63, 422, 436 f., 457, 465 f.
  phosphorus, 196–207; growth ratios, *200*
  synthesis, 152–4
RNA-P, 206–7
Rods (Stäbchen), 21

Sarcoblasts, 384 ff., 393, 394–6
  cross-striated, 399–400
  cytodifferentiation, 404–7
  differentiation in vitro, 403–4
  fibril stage, 398–9
  growth, 394–5, 402–3
  maturation, 400–1
  nuclear differentiation, 315–16, 401–2
  prefibril stage, 397 f.
  redifferentiation, first steps, 403
  transformation, 407
Sarcolemma, 346–53; sarcolemmal collagen, 351; separation from myofibrils, 353
Sarcoma 180 cells, 88, 92, 93–4, *98,* 495
Sarcomere, 355–6, *356,* 360, 362
Sarcoplasm, 365–6, 389
Sarcoplasmic matrix, 372
Sarcoplasmic reticulum, 369–72
Sarcosomes. *See* Mitochondria
Schiff reagent, 233, 235, 254
Schultz cholesterol method, 251–3, 254
Schwann cells, 12
Sebaceous cells, 323; electron microscopy, 324
Secretion: bodies, 330; and Golgi complex, 320–34; granules, 322, 420; morphology of, 305–36
Selective vulnerability to infection, 483
Sex skin, 474; barrier value, 474 f., 477
Silver: mature vs. immature collagen, 508; reduction of, 507–8; reticulin vs. collagen, 507–8
Skin: barrier value, 474 f., 477; electron microscope studies of, 30; human abdominal, collagen fibrils, *507*
Solubility, of collagen, 509–12
Specificity tests, for demonstration of sulfhydryls and disulfides, 270
Spermatid, developing, 322
Spreading reaction, behavior of, 489
Squamous epithelia, 32
Staining: methyl green, *126,* 129, *146,* 147–8; Nile blue sulfate, 254, 315, 436; phosphatides, 239–40
Starvation granules, 420
STH, 278
Steroid-producing glands, 251–3
Steroids, 241–3
Striated muscle, 346–8; cytology, 343–73; regeneration, 381–411
Striation, cross, 399–400
Striped muscle. *See* Striated muscle
Sudan black, 236–9, 310–13, 314 f., 318 f.
Sudanophilia, 236–9, 244–5
Sulfhydryls
  demonstration, 269–70, 271–2; specificity tests, 270
  distribution, 271–2
  protein-bound, 266–8
Sulfur mustards, 104
Sulfur-rich proteins, 9, 272–6
Swelling
  of cell: pathological, 417–38; and potassium content, 426; and respiration, 426
  of collagen, 512
Swelling, cloudy. *See* Cloudy swelling
Syncytium, human placental, 11

TCA, 278
Terminal bars, 29, 31–2, 35
Tetrazolium salts, 269–70
Thioguanine, 95, *96,* 100, *100,* 102, 104
Thiouracil, 211–12
Thymine, 122, 175 ff.
Thyroid gland, 13; origin of secretory granules, 332 f.
Tissue: adipose, 245–8; differentiation, 407; neoplastic, 88
Tissue culture cells, 433–8; cytoplasm, 435 f.; mitochondria in, 433–4; muscle, 386, 388–90, 403–4; poliomyelitis, 426–7; purine-treated, mitosis in, 84–107; swelling, 433–5
Toluidine blue, 491 ff.
Tonofibrillae, 31 f.
Tradescantia, 108
Trypan blue, 388 ff., 393

Ultraviolet microscopy, 9
Uracil, 176 ff.
Uridine, 207
Urodeles, 408

Vaccinia, 487 f., 494
  behavior of, 493

  infected cells, Feulgen reactions, 457
  virus, 452 ff., 456, 458, 487 f., infections, 457
Vacuolar degeneration, 423–4
Vacuoles, 47; swelling in tissue cells, 418–33
Vacuolization of cells, 417–38
Virus
  bacteria, 181
  cytoplasmia, 448–60; pox group, 451–8; psittacosis group, 448–51; rabies, 458–60
  ectromelia, 452
  herpes simplex, 463–4
  human skin papilloma, 460–3
  influenza group, 464–8
  intranuclear, 460–8
  plant, 181
  vaccinia, 452 ff., 456, 458, 487 f.; infections, 457
Vulnerability: ground substance, 497; to infection, 483

Xanthine, 89

Zymogen granules, 13, 329 f.

Plates

1. Section through the apical borders of two cells from the membranous portion of a guinea pig's yolk sac. The specimen was obtained at about the middle of pregnancy. Microvillous projections extend into the lumen of the yolk sac cavity. Some of these projections are long and exhibit bulbous terminal enlargements; others, particularly near the junctions between cells, are short and delicate. Palade's fixation. × 5,000.

2. Section through the border of a yolk sac cell similar to those illustrated in Figure 1. In addition to the slender microvilli, the plasma membrane is occasionally reflected into the interior of the cell to form canaliculi. These frequently become dilated and form vacuoles. Palade's fixation. × 15,000.

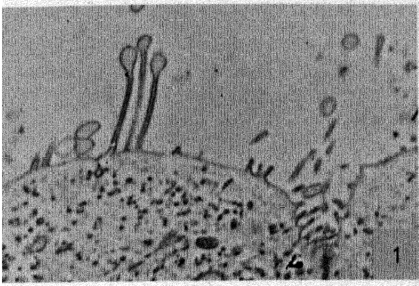
3. Section through part of a glial cell from the region of the area postrema of a rat. At the arrow, the plasma membrane can be seen to turn inward and form an invaginated, microlabyrinthine space. This cell is one of the varieties of protoplasmic astrocytes, all of which exhibit extensively infolded surface membranes. Palade's fixation. × 15,000.

4. Section through the thin portion of a human chorionic villus obtained at term. Microvilli project into the maternal intervillous space, illustrated at the top of the figure. Compare their appearance with those in a specimen obtained earlier in pregnancy, illustrated in Figure 11. Beneath the syncytium are two basement membranes enclosing a connective tissue space in which collagenous fibrils may be seen. The element at the base of the figure is a portion of an endothelial cell from the wall of a fetal capillary. Palade's fixation. × 15,000.

5. Section through the apical margin of a uterine cell in the maternal placenta of a horse. The specimen was obtained at the third month of pregnancy. In addition to slender, branched microvilli, these cells possess cilia. The constituent fibrils of these structures are illustrated both in cross and longitudinal section. In the basal bodies or rootlets of the cilia, a striated configuration is evident. Such basal striations have been observed previously by Fawcett (see Chapter 3) in lower animals but have not been seen before in mammalian cells. Palade's fixation. × 30,000.

6. Section through the junction between the fetal chorion (above) and the uterine epithelium (below) from a horse's placenta. The projecting microvilli of both cells form an interdigitating union. Palade's fixation. × 15,000.

7. Section through the apical margins of two cells from a human uterine gland located in the decidua vera of an individual 9 weeks pregnant. Apical microvilli project into the gland's lumen. Similar microvilli have been seen in other secretory epithelia. Their occurrence in such locations casts doubt on the hypothesis that they provide merely an extended surface for absorption. Palade's fixation. × 15,000.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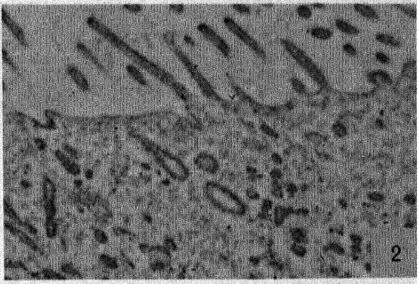
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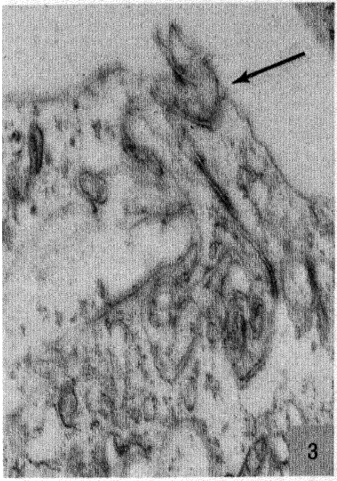
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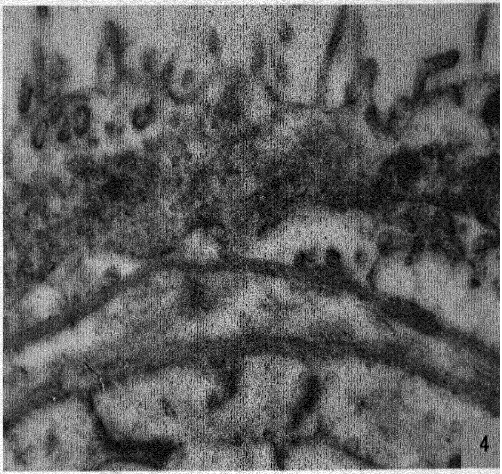
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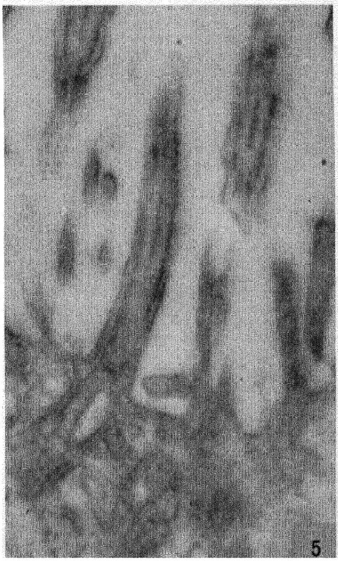
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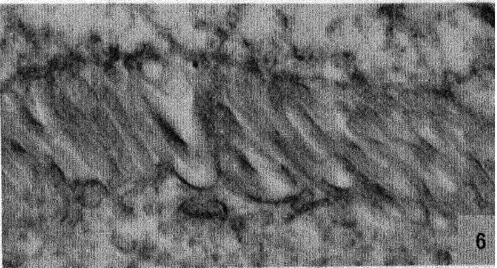
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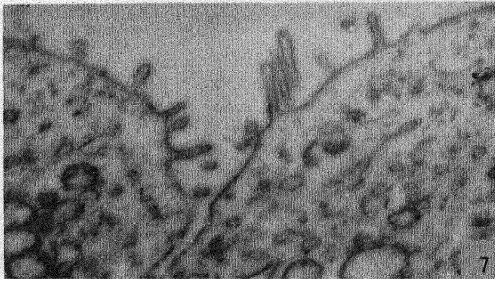
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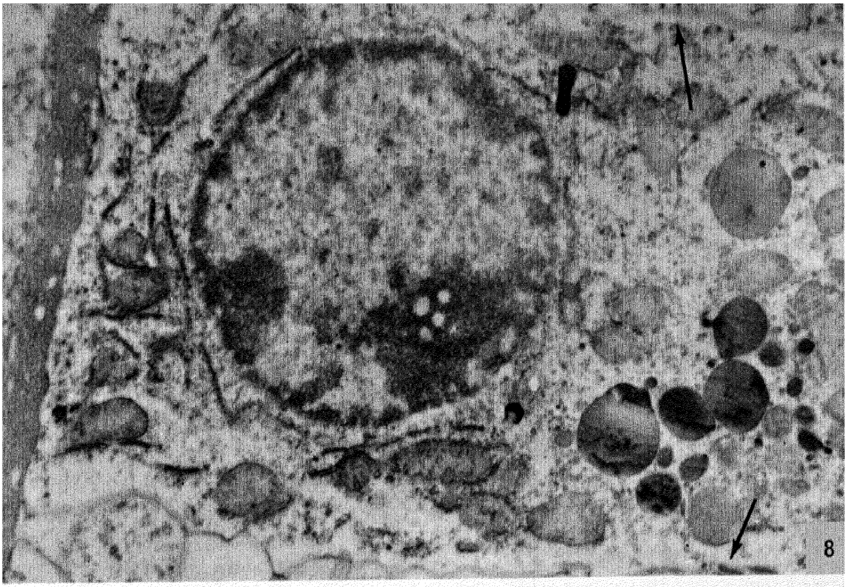
8 and 9. These sections represent two cells from the yolk sac epithelium of the guinea pig. Figure 8 is from an animal killed during mid-pregnancy; Figure 9 is from an animal killed at term. Marked differences characterize the mitochondria, ergastoplasm, basement membranes, and other structures at these two periods. The lateral margins of the cells frequently project into a neighboring cell in the manner shown at the arrow near the upper margin of Figure 8. Local thickenings of the marginal plasma membranes occur at adjacent places on contiguous cells (arrow, lower margin Figure 8). Palade's fixation. × 10,000.



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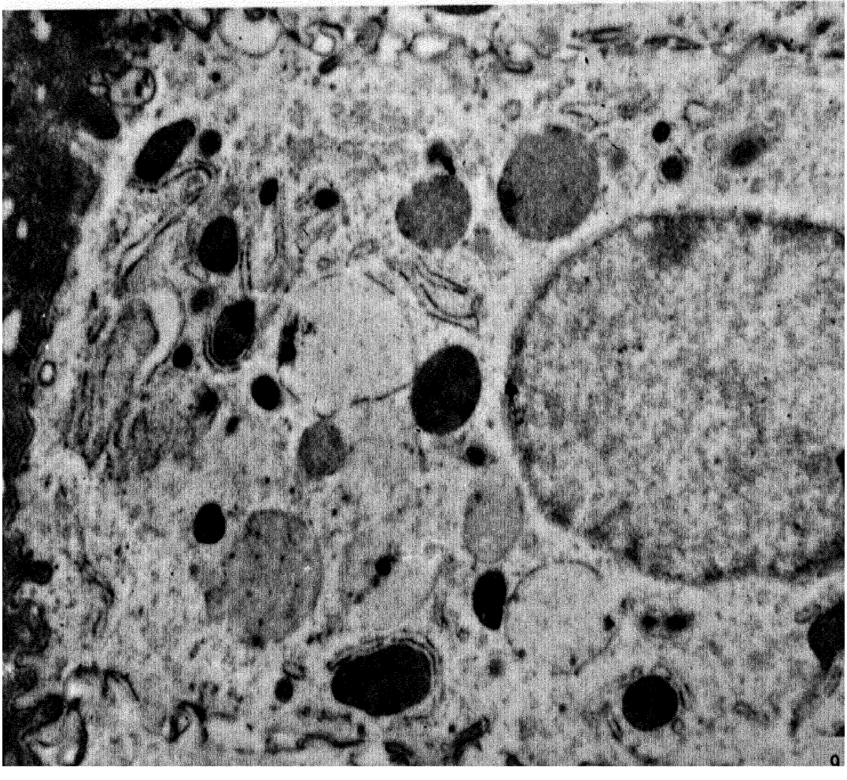

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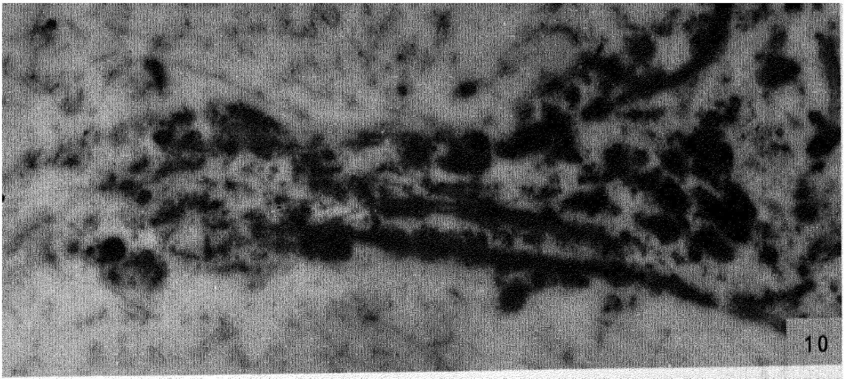
10. Section through a cell process of a fibroblast from the decidua vera of a human uterus obtained at the 9th week of pregnancy. Along the margins of the pseudopodium, a definite cell wall is visible, but at its tip the cell wall is indistinct and there seems to be an intermingling of the substances inside and outside of the cell. In living cells the margins of such pseudopodia have been observed to vary in their distinctness from moment to moment. Palade's fixation. × 15,000.

11. Section through the syncytial trophoblast covering a human chorionic villus from a placenta of 9 weeks gestation. The apical surface is irregular. Branched and bulbous microvilli project into the intervillous space. In addition, larger promontories also protrude into the space from the surface of which smaller microvilli arise. Compare the irregularity of this surface with that from the term placenta illustrated in Figure 4. Scattered throughout the cytoplasm are numerous spherical vesicles bounded by a dense, granular membrane. These vesicles contain an amorphous substance of slightly greater density than the maternal plasma. They occur in the portion of the cell which is basophilic. This fact, together with the similarity of their granular membranes, suggest that they represent ergastoplasmic sacs dilated by the presence of a colloidal protein. Palade's fixation. × 12,000.

12. Slightly tangential section through the wall of a thyroid follicle from a rat. The follicular colloid is shown at the upper left-hand corner of the figure. Small microvilli project into the follicular lumen. Within the cytoplasm of the cells, irregular spaces are bounded by a dense granular membrane. These spaces contain an amorphous, colloid-like substance of varying density. These are equivalent to the colloid-containing follicles of light microscopy, and the colloid droplets are interpreted as forming within the lumens of ergastoplasmic elements. Palade's fixation. × 12,000.

13. Section through the basal portion of a cell from the uterine gland of a pregnant sow. The edge of the nucleus appears at the top of the figure; across the bottom is the basement membrane and the subjacent connective tissue. Within the cytoplasm, elongated strands of granular ergastoplasmic elements course in various directions. Some of these are dilated at their ends, and appear to form small vesicles, a cluster of which is visible in the right center of the field. The lumens of these vesicles exhibit various densities. Such appearances have been correlated with the formation of secretory material. Palade's fixation. × 15,000.

14. Section through a human chorionic villus of 9 weeks gestation. The field illustrates the wall of a fetal capillary in the stroma of the villus. The central and right-hand portions of the figure depict part of a fetal fibroblast which contains irregular ergastoplasmic elements, the lumens of which are filled with a moderately dense substance. We interpret the contents of the ergastoplasmic sacs as indicating the secretion of protein for the growing connective tissue elements. Palade's fixation. × 20,000.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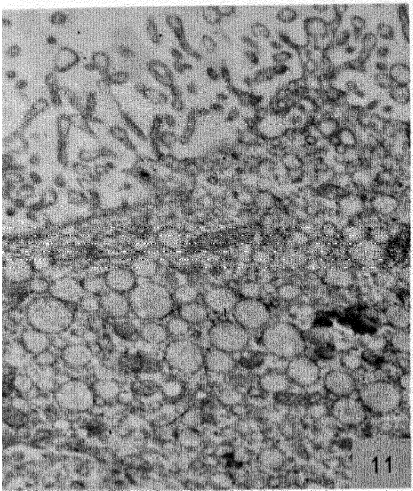
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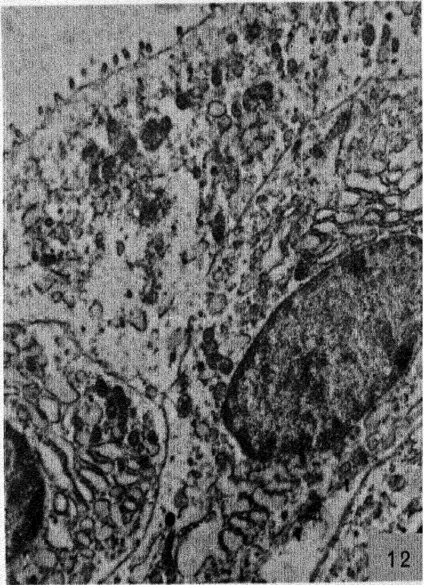
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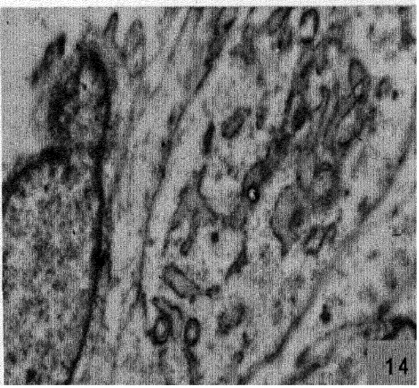
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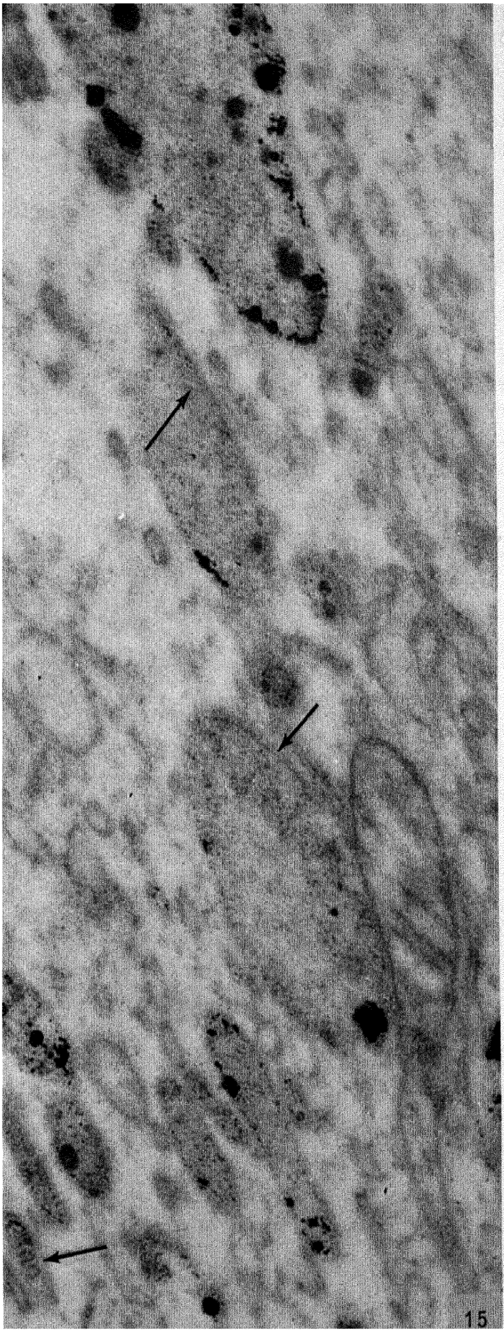
15. Section through a hepatic cell from a rat which had received silver nitrate in its drinking water for 12 months. Occasional aggregates of silver occur in the parenchymal cells of such animals, although most of the silver is retained by the basement membranes and by macrophages. In these parenchymal locations, the granules are segregated within bodies which resemble mitochondria in size and density, and which sometimes exhibit double outer membranes and linear arrays suggestive of the mitochondrial membranes (arrows). Palade's fixation. × 25,000.

16. Section through the apical portion of a pancreatic cell from the same animal from which Figure 15 was derived. The top portion of the figure depicts the acinar lumen. Beneath it are several zymogen granules. A large structure containing silver deposits occupies the lower center of the field. It contains a clumped, membranous formation, the elements of which are similar to the internal mitochondrial membranes. Palade's fixation. × 30,000.



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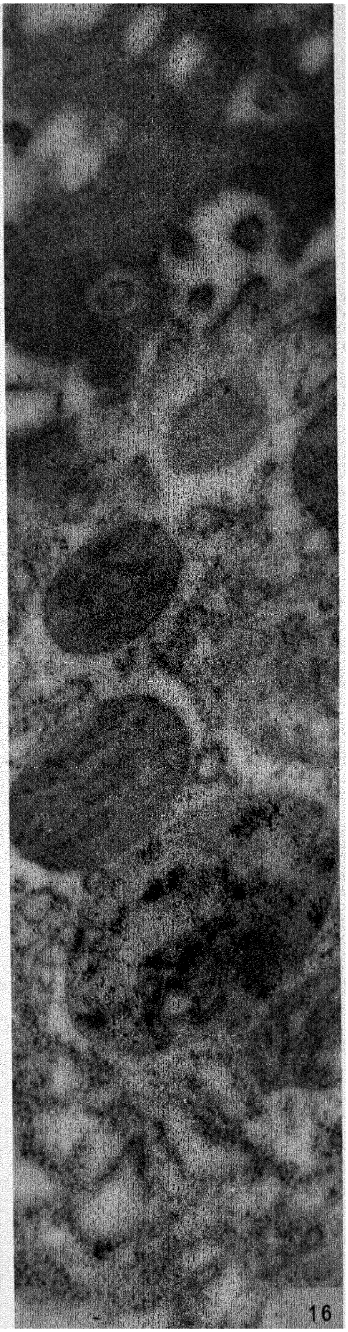

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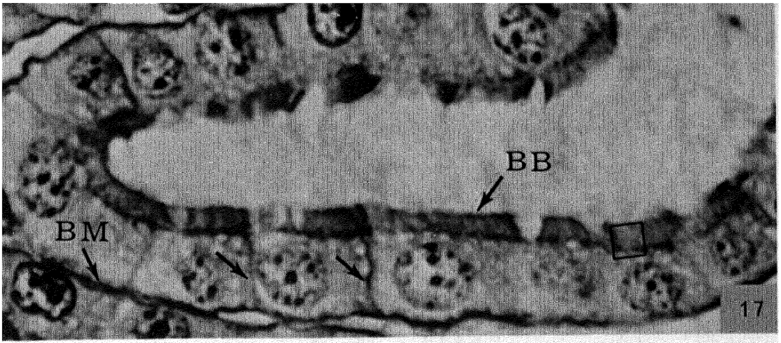
17. Photomicrograph of a proximal convoluted tubule of a frog nephron stained with the periodic acid-Schiff reaction and counterstained with hematoxylin. The well-developed brush border (BB), and the basement membrane (BM) have a positive staining reaction indicating the presence of a carbohydrate containing component. Note also at the arrow, the staining of the cell boundary. An area such as that enclosed in the rectangle is shown in the electron micrograph, Figure 21. × 1,200.

18. Photomicrograph of intestinal epithelium of a cat showing a prominent striated border (SB). An area of the border such as that in the rectangle is shown in an electron micrograph in Figure 19. Heidenhain's Azan stain. × 900.

19. Electron micrograph of a small area of the striated border of rat intestinal epithelium. The border processes or microvilli have a protoplasmic content that is somewhat more dense than the subjacent cytoplasm. Micrograph contributed by Dr. Sanford Palay and Dr. Leonard Karlin of the Department of Anatomy, Yale University Medical School. × 67,000.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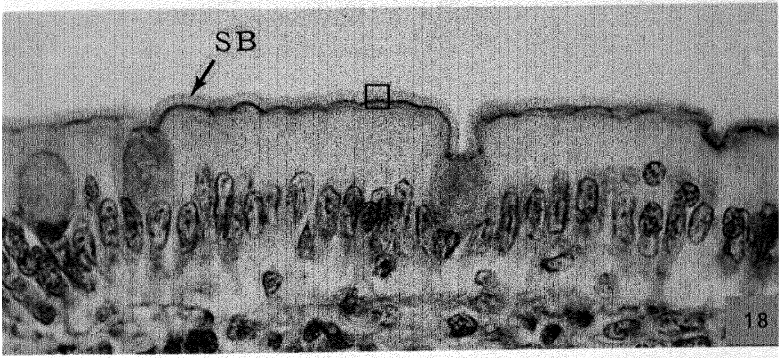
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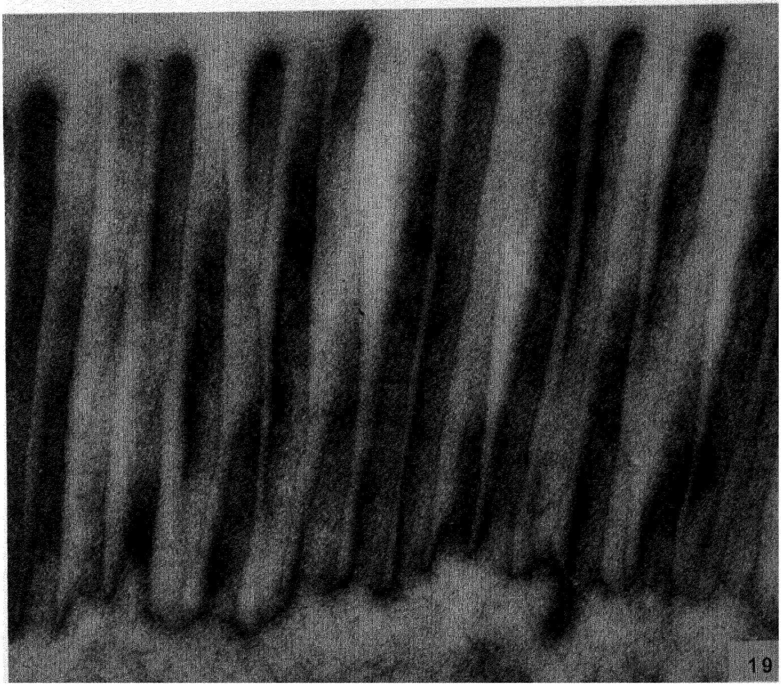
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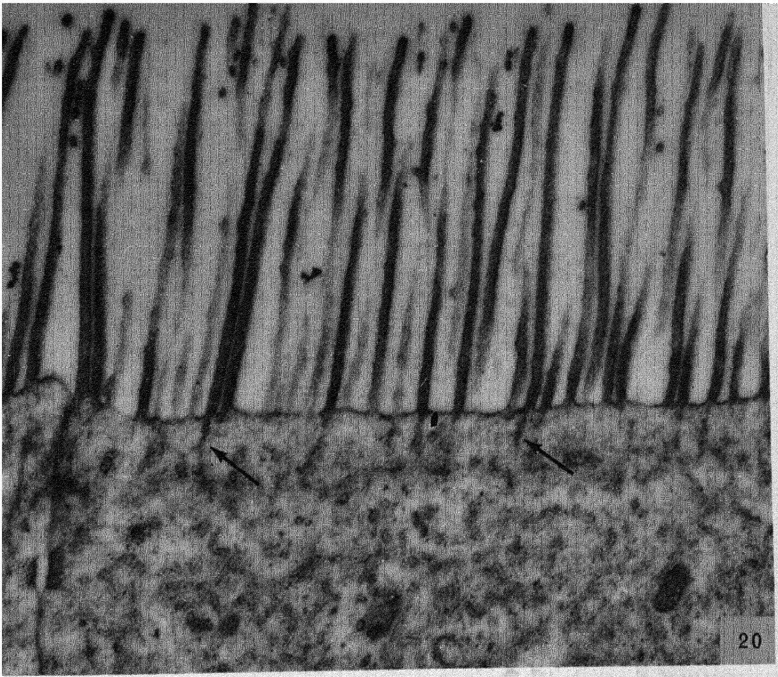
20. Vertical section of the striated border of the intestinal epithelium of *Triturus viridescens*. The processes are long and slender, and the dense protoplasm in their interior extends downward a short distance into the cytoplasm (see arrows). × 43,500.

21. Vertical section of the brush border of the proximal tubule of *Rana pipiens*. The border processes are closely packed with no intervening free space. Tubular invaginations of the cell surface (C) arise from the depths of the interstices between the border processes and run downward into the apical zone of cytoplasm. × 47,500.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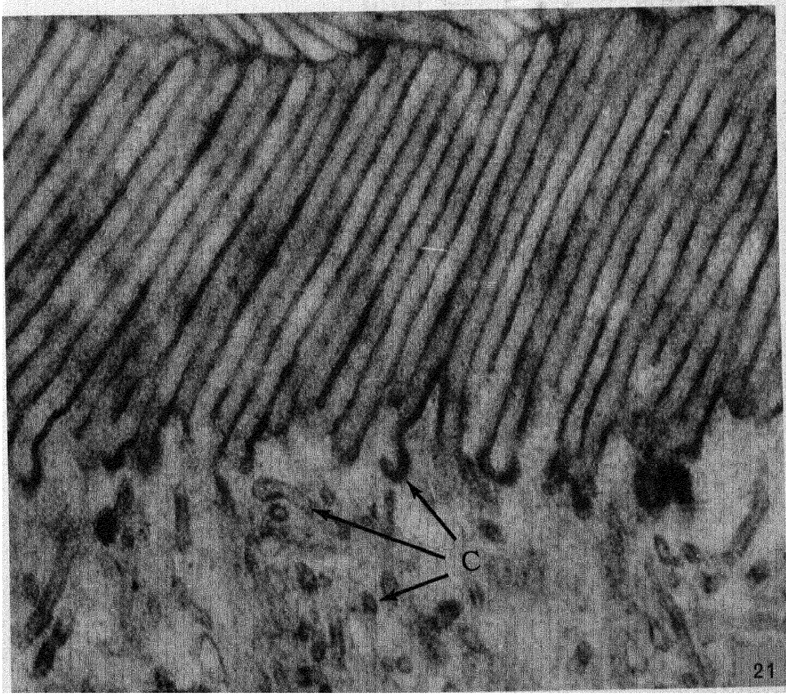
C
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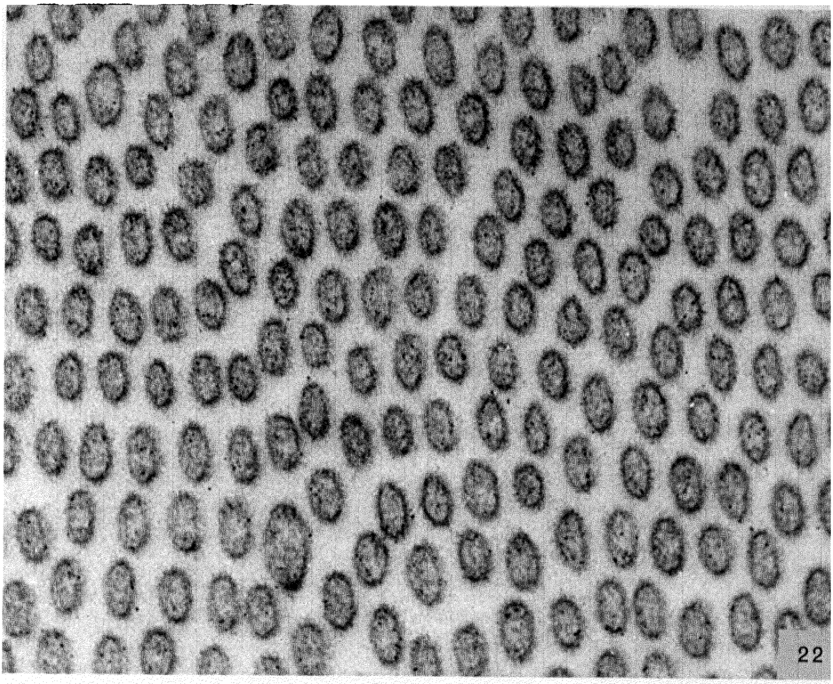
22. Horizontal section through the intestinal striated border of *Triturus viridescens.* The border processes in cross section are round or oval and have a granular protoplasmic content and a distinct limiting membrane. They appear to be free processes with no surrounding amorphous matrix. × 57,800.

23. Horizontal section through the brush border of the proximal segment of the frog nephron. The border processes appear in cross section as light circular areas outlined by a moderately dense homogeneous substance which occupies the interstices. The processes seem to be embedded in an amorphous matrix. The appearance depicted here is probably the basis of erroneous earlier reports to the effect that the brush border is comprised of ducts or tubules. × 61,000.



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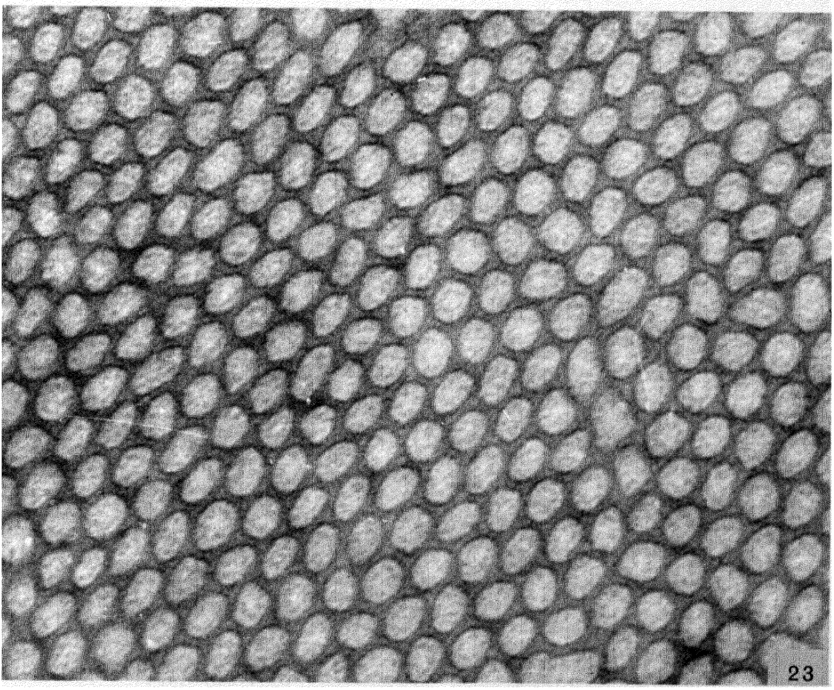

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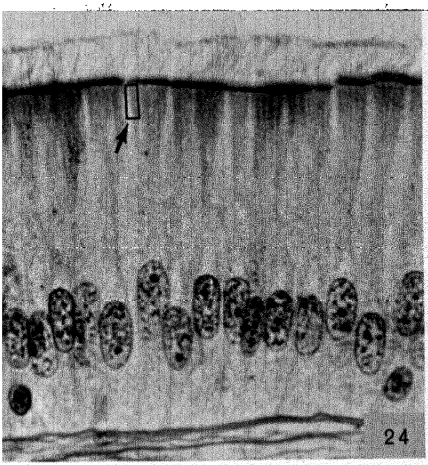
24. Photomicrograph of ciliated epithelium from the typhlosole of the mussel *Elliptio complanatus*. The ciliary apparatus consists of the cilia, a row of darkly stained basal corpuscles, and a cone of rootlets which run downward from the basal bodies into the cytoplasm. An area such as that in the rectangle is presented in Figure 28. Chrome alum hematoxylin phloxine. × 800.

25. Electron micrograph of a cilium from the bryozoan, *Pectinatella magnifica*. There are nine double filaments around the outside and a pair of single ones in the center. The filaments have a dense rim and a less dense interior and therefore have the appearance of slender tubes. × 150,000.

26. Structures at the base of a cilium of *Pectinatella*. At least three cross-striated rootlets arise from the large hollow-appearing basal corpuscle. Two of these run horizontally beneath the cell surface and one extends vertically downward into the cell body. × 37,800.

27. A rootlet fiber from the ciliated epithelium of the typhlosole of *Lumbricus terrestris*. The major period is 636A and at least five bands are distinguishable within this period. × 100,000.

28. A pair of cross-striated ciliary rootlets from *Elliptio*. The fibers have a period of about 700A. The broad band seems to correspond to a group of 3 bands resolved in the fiber shown in Figure 27. The narrower band has its counterpart in the distinct narrow band in the rootlet of *Lumbricus*. It is believed that the fibers are essentially the same in the two species but more than two bands are seldom resolved in the *Elliptio* material. × 57,800.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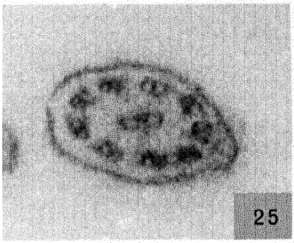
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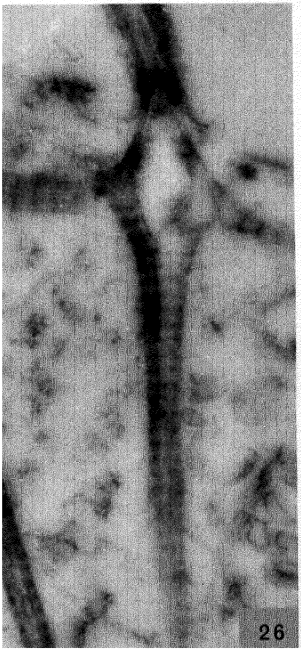
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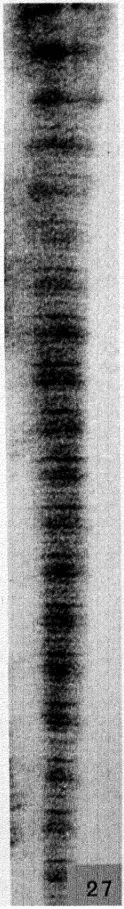
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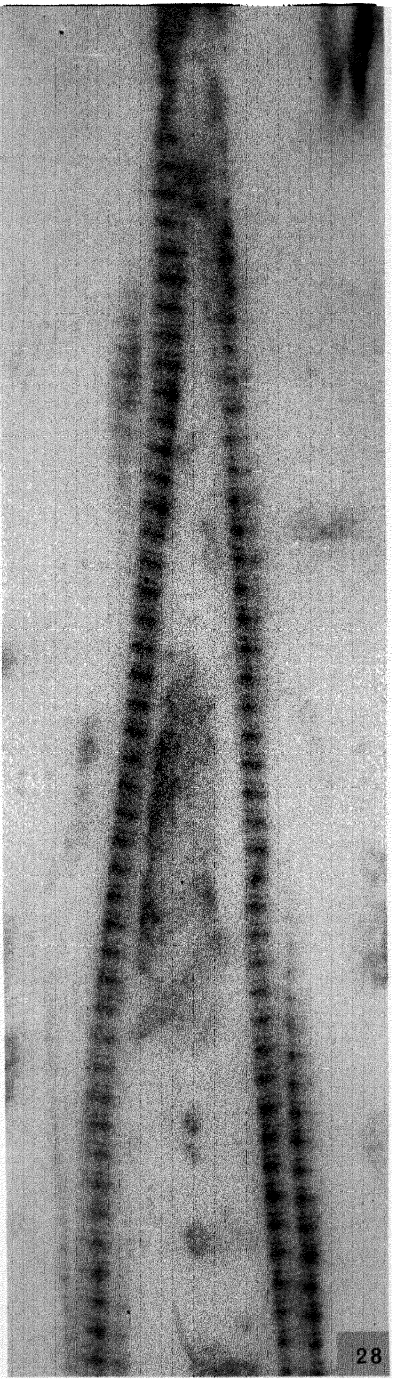
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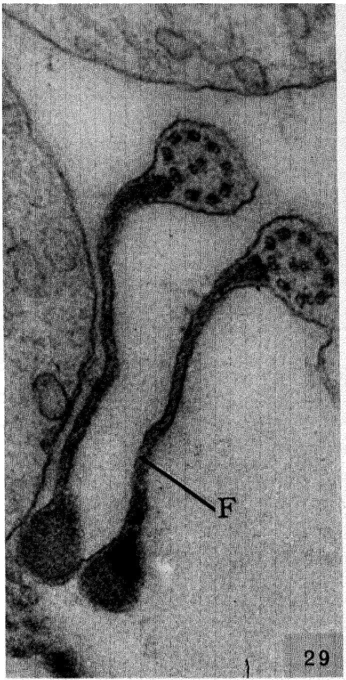
29. Cross sections of two sperm tails of *Bufo arenarum* showing the same number and arrangement of filaments as are found in cilia. The sperm flagellum in this species has a broad fin (F) of dense material with a thickening along its free margin. The fin is enclosed in an extension of the flagellar membrane. × 53,000.

30. Cross sections of sperm flagella of *Triturus viridescens.* The dense material which runs in the free edge of the fin in this species is trifoliate in section. × 20,000.

31. Longitudinal section of a sperm tail of *Macacus mulatta.* The bundle of longitudinal filaments is visible in the center with the dense double strands of the circumferential wrapping on either side. × 53,000.

32. Junction of the intermediate piece (I.P.) and principal piece (P.P.) of a monkey spermatozoön showing the "ring centriole" (R) at the junction, the mitochondria (M) of the intermediate piece, above, and the so-called cortical helix (CH) below. The double strands of the latter branch and anastomose and are held together by two longitudinal bands which run along opposite sides of the flagellum. × 35,000.

33. Transverse sections of several sperm tails showing the dense wrapping interposed between the longitudinal fiber bundle and the flagellar membrane. The opposite thickenings of this layer (arrows) are sections of the two longitudinal bands which hold together successive turns of the wrapping. × 42,000.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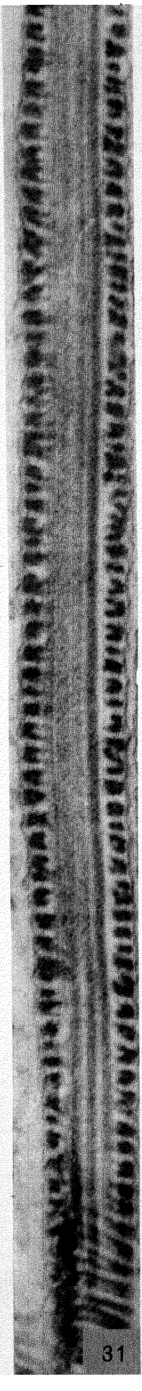
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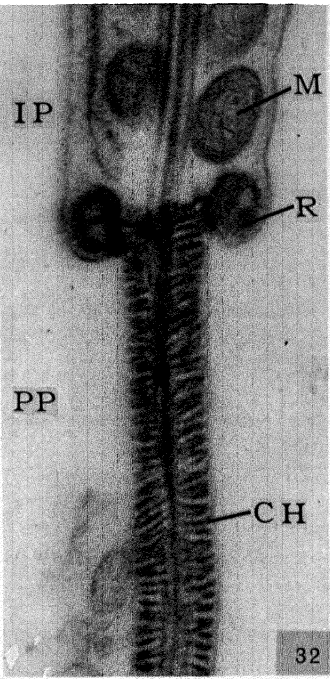
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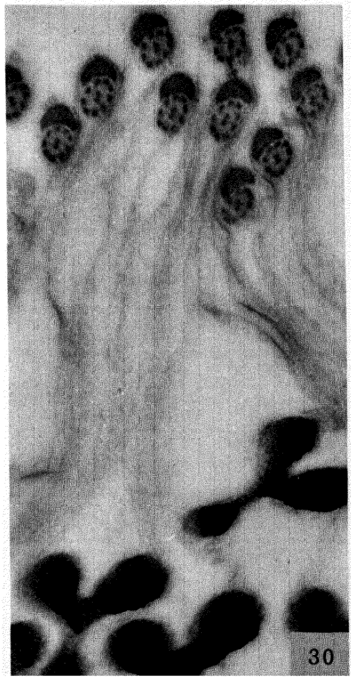
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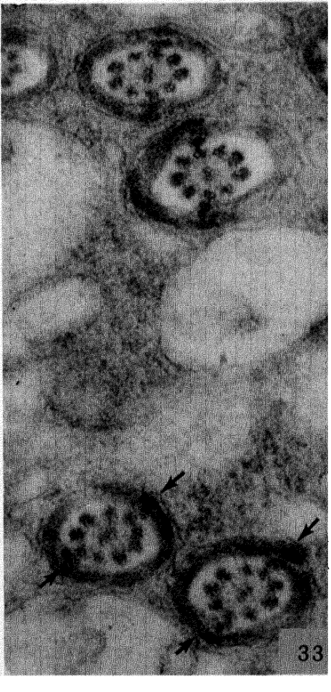
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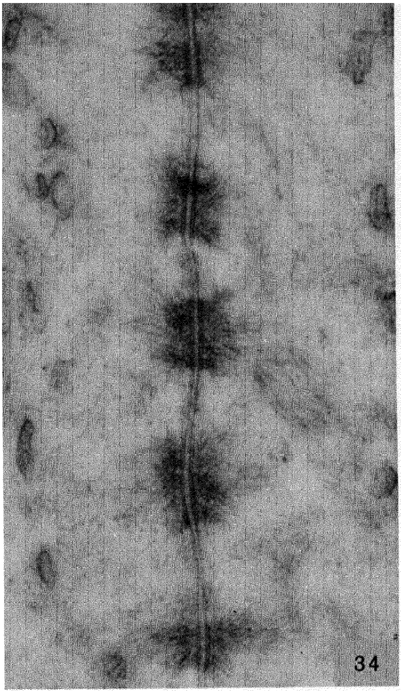
34. A portion of the interface between two epidermal cells of *Ambystoma punctatum* showing a series of "desmosomes," corresponding to the "intercellular bridges" of light microscopy. These consist of a local thickening of the opposing cell membranes, and the protoplasm immediately adjacent to these thickened areas is unusually dense. Tufts of fine filaments radiate from the dense material into the cytoplasm of the cells. Micrograph contributed by Dr. K. R. Porter, Rockefeller Institute for Medical Research, New York. × approximately 35,000.

35. A pair of desmosomes (at the arrows) on cells of the Lucké renal carcinoma of the frog, *Rana pipiens*. There is marked thickening of the cell membranes and an increased density of the adjacent cytoplasm. The filaments, seen in Figure 34, are present, but inconspicuous. × 46,500.

36. Desmosome (at arrow) on the interface between two cells in the parietal layer of Bowman's capsule in normal frog kidney. × 40,000.

37. A longitudinal section of a "terminal bar" (at arrow) on the contact surfaces of two cells of a renal carcinoma of the frog. A sharply defined band of dense material is found on either side of the thickened plasma membrane. × 35,000.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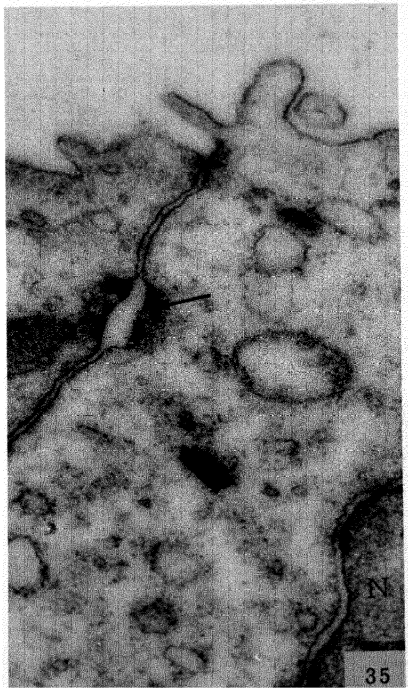

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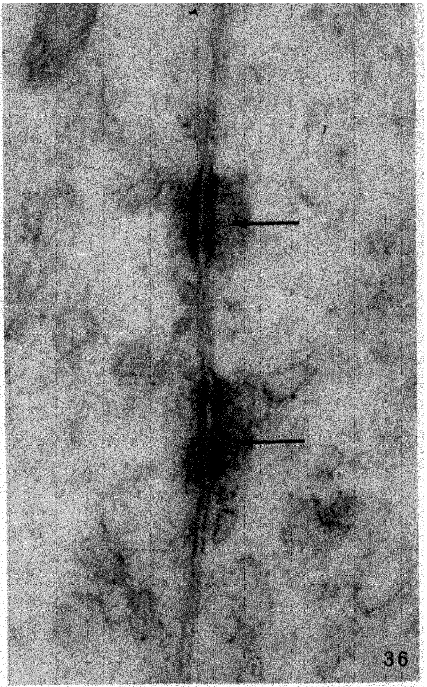
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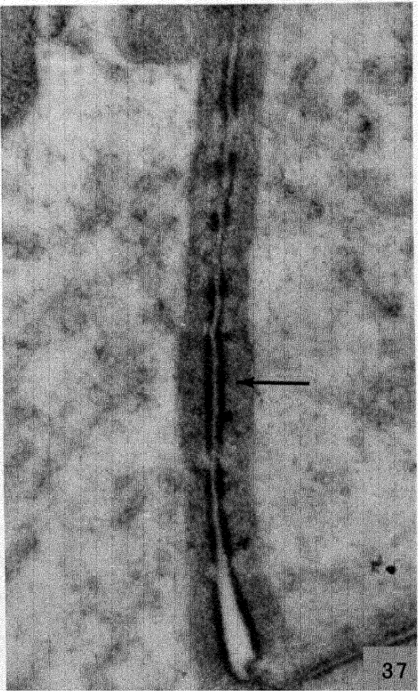
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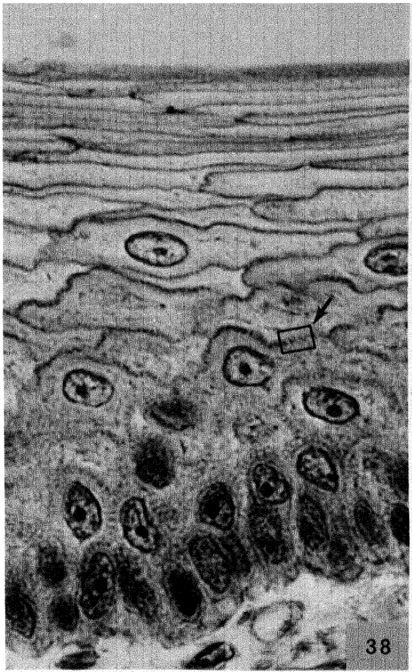
38. Photomicrograph of stratified squamous epithelium of the gingiva of *Felis domestica.* An area such as that enclosed in the rectangle is shown in the electron micrograph of Figure 39. × 800.

39. The cell surfaces in the stratum spinosum have a regular pattern of alternating ridges and grooves that fit closely together without intercellular spaces or bridges. Desmosomes are irregularly distributed over the surface corrugations (at arrows) but are difficult to see because of the accumulation, at the periphery of the cell, of dense fibrous material presumed to be keratin. × 46,000.

40., 41., 42. The interdigitating surface corrugations of squamous epithelial cells of the tongue which have been pulled apart slightly in specimen preparation. The dense feltwork of fibers immediately beneath the plasma membrane apparently stiffens the ectoplasmic layer, and the surface ridges therefore persist even after exfoliation of the cells from the surface of the epithelium. × 48,000, 27,000, and 46,000.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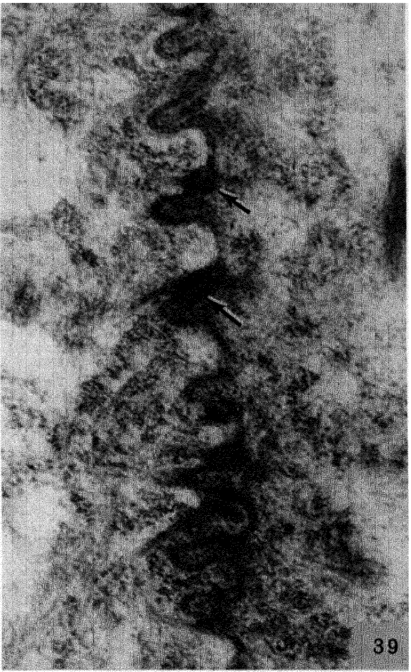
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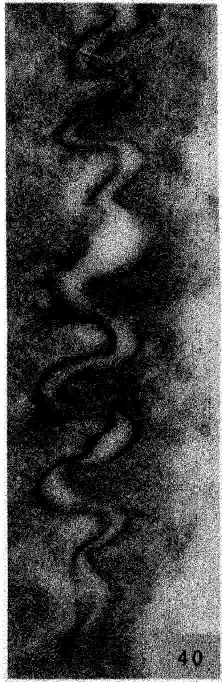
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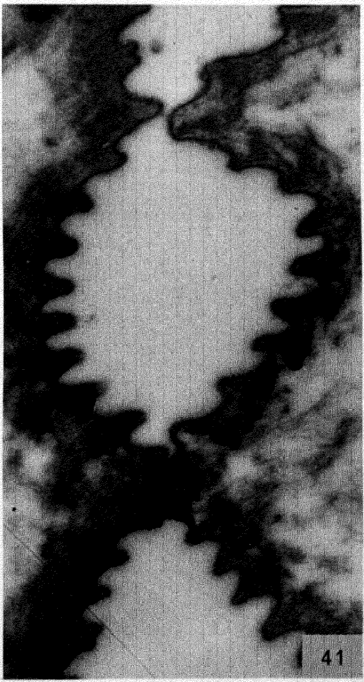
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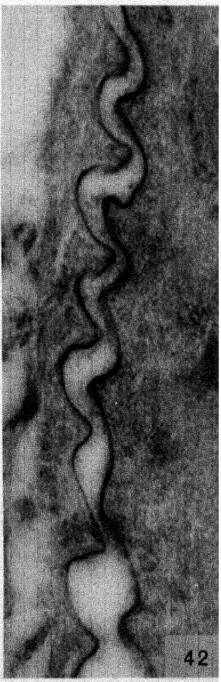
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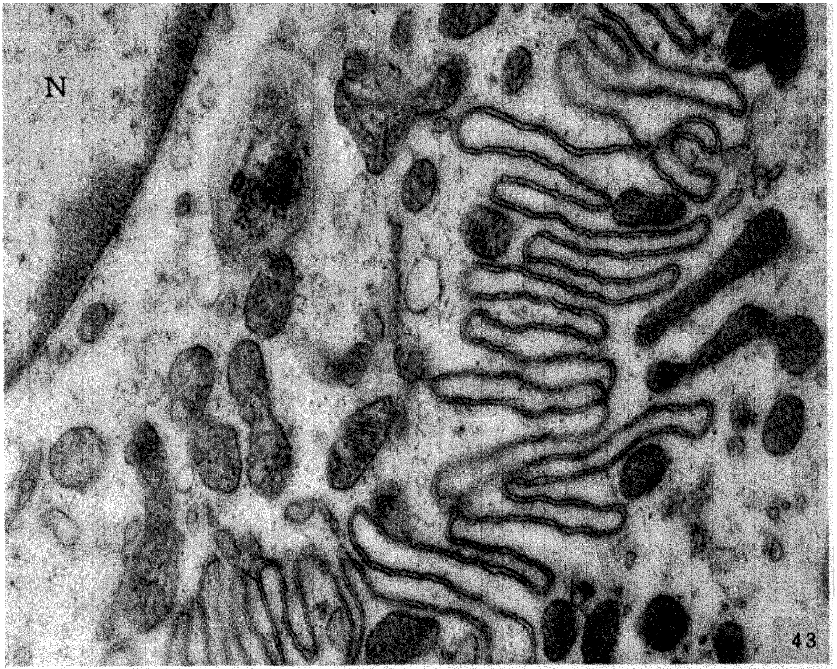
43. A portion of the junction between two cells in the distal convoluted tubule of the nephron of the frog, *Rana pipiens*. The confronted plasma membranes run a serpentine course which greatly extends the surface of contact between adjacent cells. The parallel dense lines representing sections of the cell membranes remain a very uniform distance apart. N, nucleus. × 20,566.

44. A horizontal section through a cell of the distal tubule at a level near the cell base. There is an extraordinarily complex compartmentation of the cytoplasm by infolding of the plasma membrane. A very large surface is thus brought into close relation with the mitochondria (M) which are concentrated in this region of the cell. × 24,225.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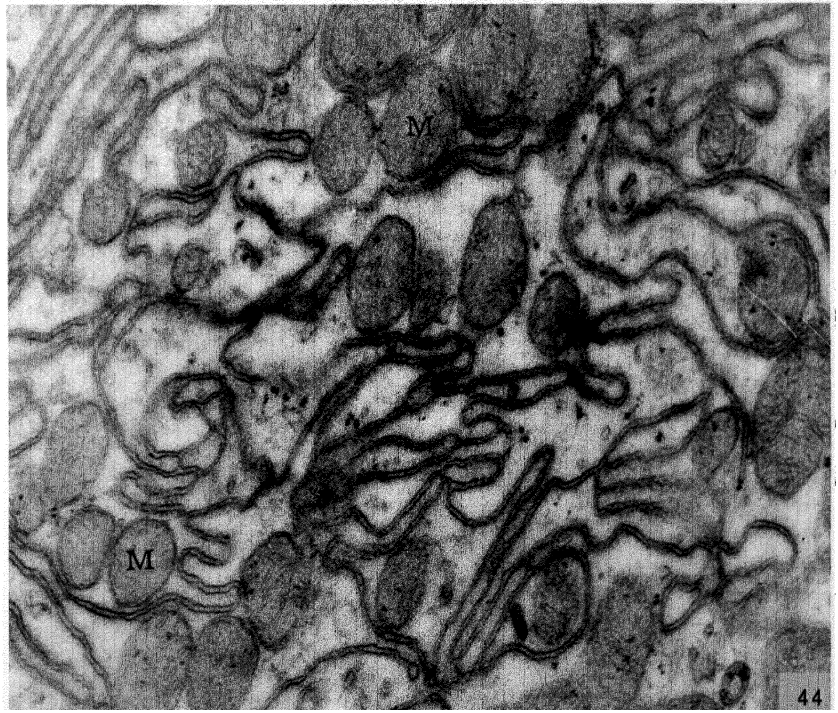
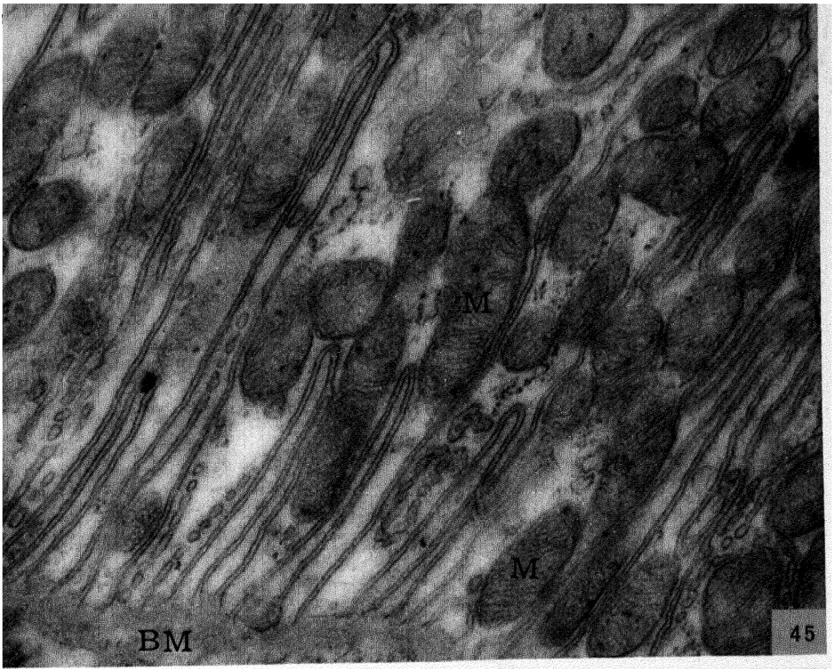

M
M
44

45. A vertical section through the base of a cell of the distal convoluted tubule of the frog kidney. The cell rests upon a homogeneous basement membrane (BM). The plasma membrane, which is closely applied to this layer, turns inward at many points and extends up into the cytoplasm forming thin folds or septa that pass between the vertically oriented mitochondria (M). × 31,400.

46., 47. Two examples of the studlike projections of the hepatic cell surface which fit into concavities in the adjacent cell in a manner suggesting that they may play some role in maintaining cell attachment. × 40,000.



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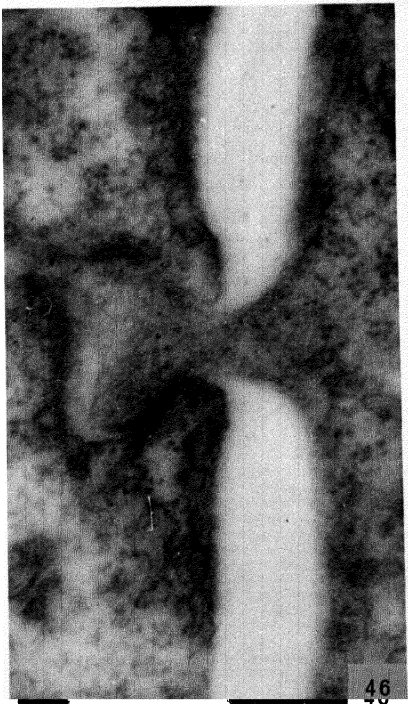

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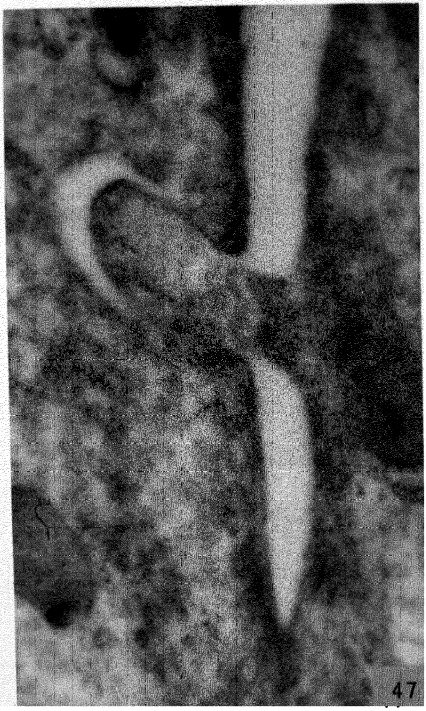


47

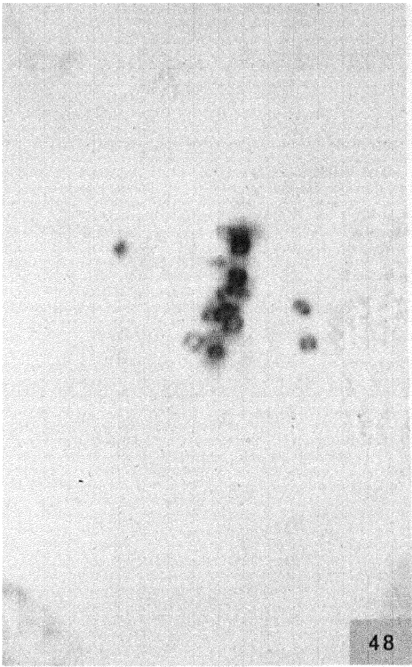
Mitotic figures in mouse tissue cultures, fixed with alcohol-acetic acid and stained with the Feulgen reaction. × 2,025.

48. Delayed metaphase figure with wide chromatid splits and three lagging chromosomes, in culture of embryonic skin treated one day with 1 mM thioguanine plus 1 mM adenosine-3′-phosphate.

49. Metaphase figure with nine lagging chromosomes, in embryonic skin culture treated two days with 0.0025 mM purine riboside.

50. Anaphase figure with chromosome fragment left at plate but attached by thin threads to polar groups, in embryonic skin culture treated with 0.05 mM thioguanine.

51. Late telophase figure with bichromatid chromosome fragment left at plate, in sarcoma 180 culture treated with 1 mM thioguanine plus 1 mM adenosine triphosphate.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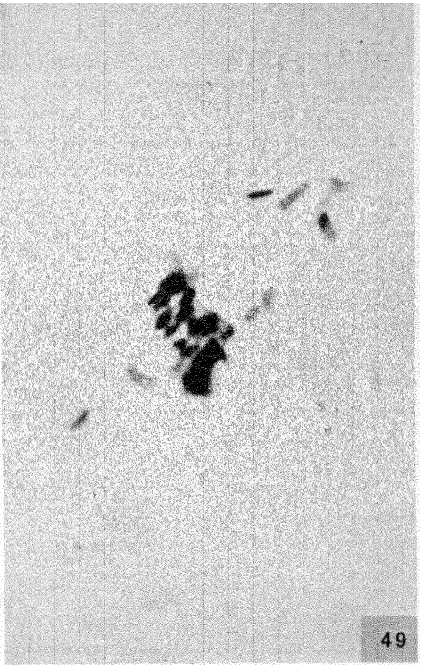
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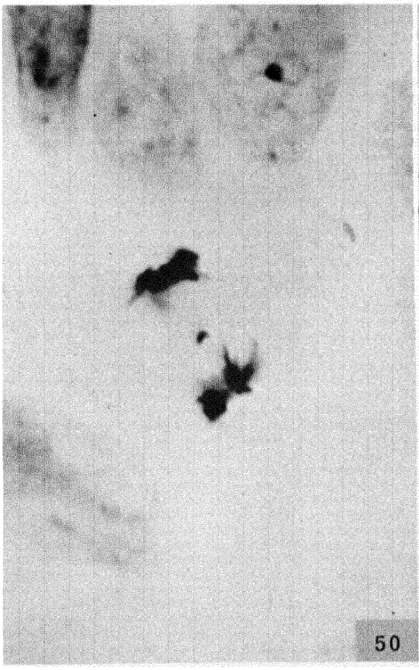
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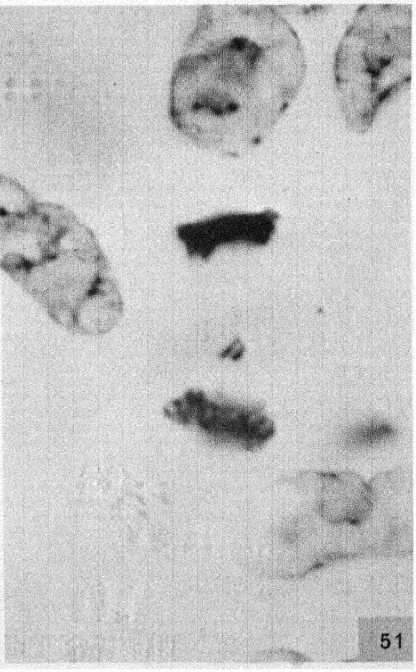
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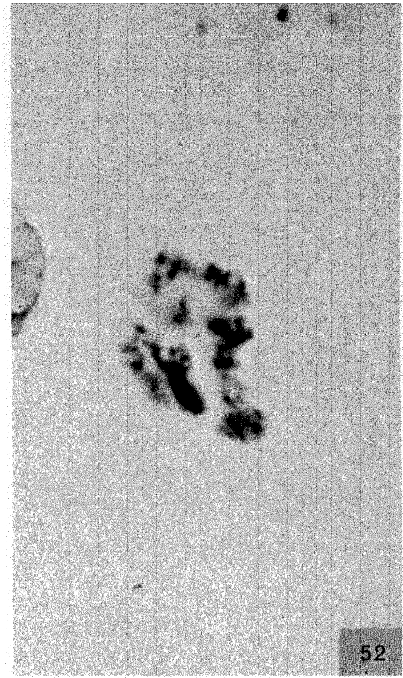
Mitotic figures in mouse tissue cultures, fixed with alcohol-acetic acid and stained with the Feulgen reaction. ×2,025.

52. Unseparated reconstruction figure of ten attached lobes, in sarcoma 180 culture treated with 1 mM 6-chloropurine plus 1 mM DPN.

53. Anaphase figure with several "sticky" bridges, in sarcoma 180 culture treated with 0.01 mM unsubstituted purine.

54. Bridged anaphase figure with several chromosome fragments, in embryo skin culture treated for one day with 0.04 mM pyridoxine-nitrogen mustard.

55. Telophase figure with three bridges and ten chromosome fragments, in embryo skin culture treated with 0.002 mM triethylene melamine.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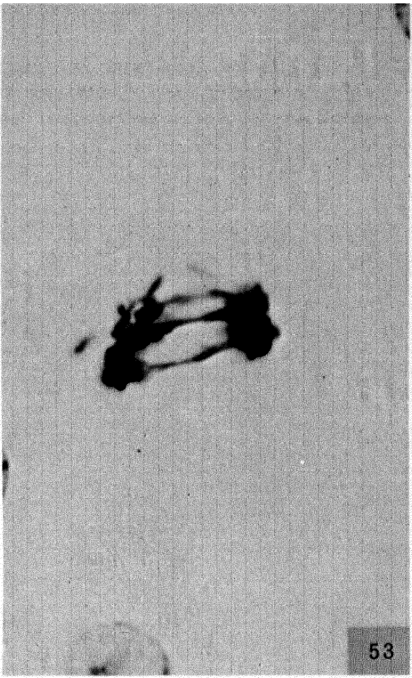
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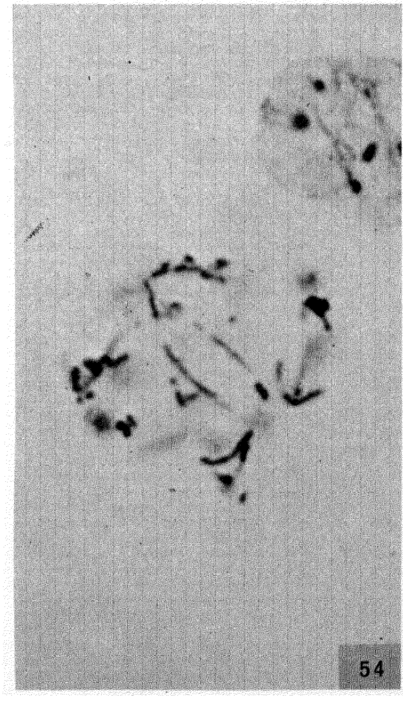
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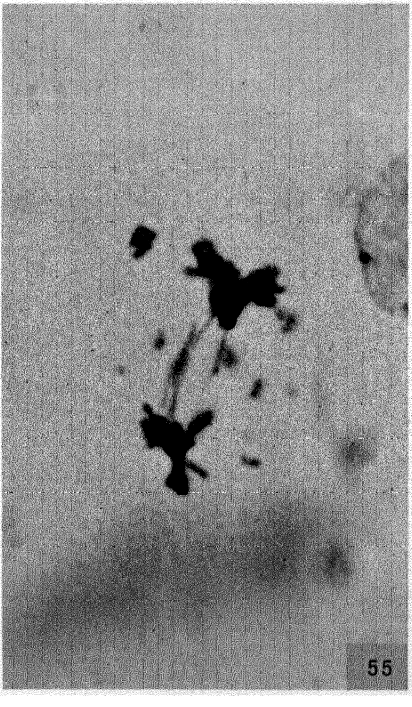
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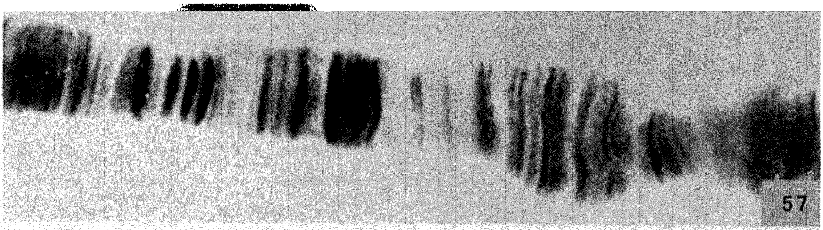
The cellular distribution of DNA and PNA as revealed by ultraviolet photography and enzymatic digestion.

57. Chromosome segment of *Chironomus,* fixed in lanthanum acetate-acetic acid. Note the heavily absorbing bands of DNA-rich material. Ultraviolet photomicrograph taken at 275 millimicra, N.A. of objective 1.7., × 930. From Caspersson [16], Figure 92.

58., 60. Ultraviolet photomicrographs of liver cells and hypothalamic (supraoptic and paraventricular nucleus) neurons respectively from a control, fed, female albino rat. Frozen-dried 4 μ sections. Wavelength 257 mμ, N.A. of objective 1.25. × 1,250.

59., 61. Ultraviolet photomicrographs of liver cells and hypothalamic neurons respectively from same blocks as in 58 and 60. Sections incubated with protease-free ribonuclease, 1 mg. per 100 ml. of citrate-phosphate buffer, pH 6.85, for 1 hour at 37° C. Photographic factors as in Figures 58 and 60. Note disappearance of the heavily absorbing cytoplasmic aggregates in both liver cells and neurons after ribonuclease digestion. Reduction of nucleolar selective absorption is most evident in Figure 61, where a faintly absorbing nucleus can be seen in the top center of the photomicrograph.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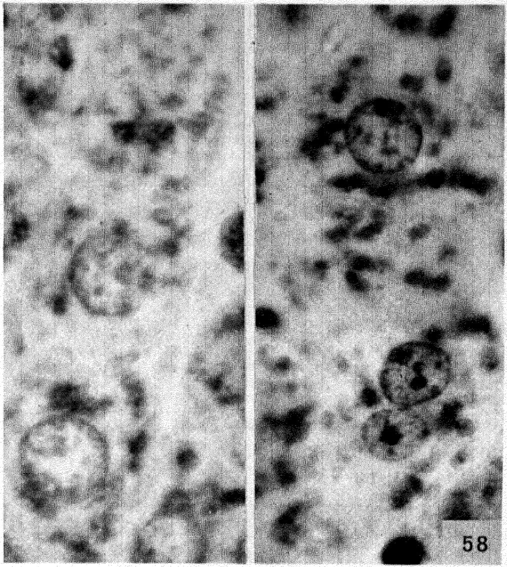
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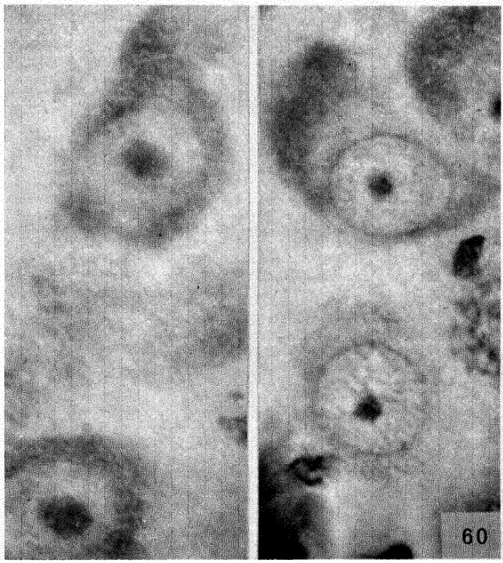
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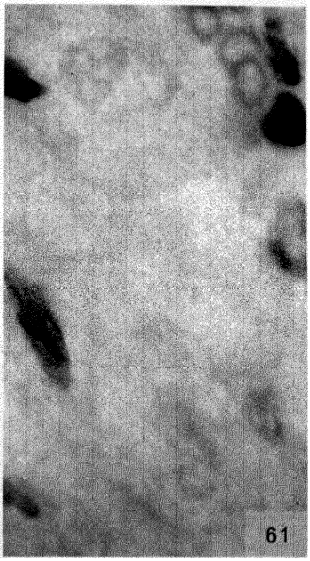
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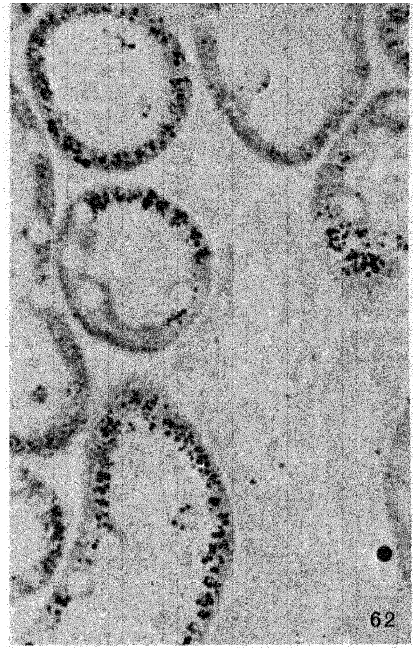
62. Kidney from a rat perfused briefly with calcium-formalin. Block then fixed, postchromated, and stained with acid hematein according to Baker's [10] method for phosphatides, × 480. Courtesy Dr. D. W. Fawcett. Many tubules lack any detectable phosphatide; others contain coalesced globules.

63. Kidney from a rat perfused briefly with calcium-formalin-dichromate. Block then treated as in Figure 62. Courtesy Dr. D. W. Fawcett. The phosphatide component of all mitochondria is stained (the reaction of the erythrocytes probably indicates hemoglobin rather than phosphatide, being positive in control preparations). G, glomerulus.

64. Mouse adrenal fixed for a week in 10 per cent neutral buffered formalin, washed, sectioned at 15 μ on the freezing microtome, and stained for 15 minutes in Schiff's reagent, × 100. The lipide droplets give an intense pseudoplasmal reaction [105].

65. Mouse adrenal fixed for a week in 10 per cent neutral buffered formalin containing 0.1 per cent hydroquinone, then treated as in Figure 64. No pseudoplasmal reaction occurs. [105].

66., 67. Mouse adrenals fixed for a week in (66) a saturated solution of ammonium sulfate and (67) a saturated solution of phosphotungstic acid, then treated as in Figure 64. In both, the lipides give a pseudoplasmal reaction [105].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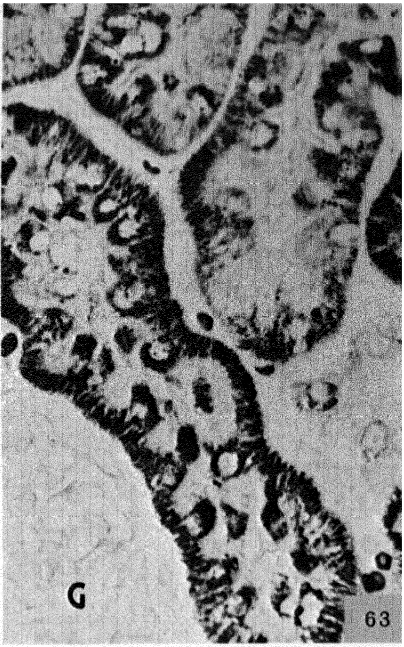

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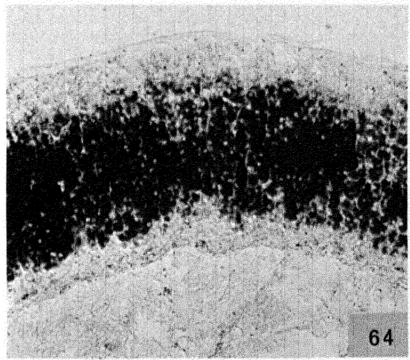
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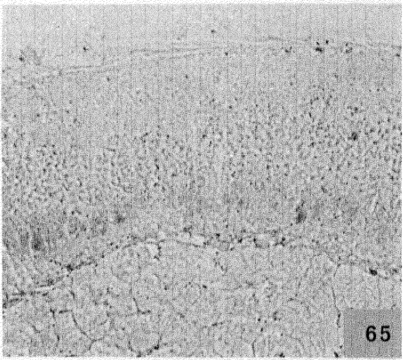
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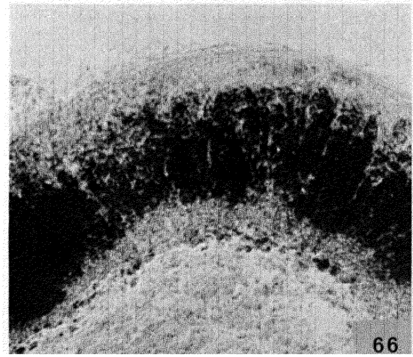
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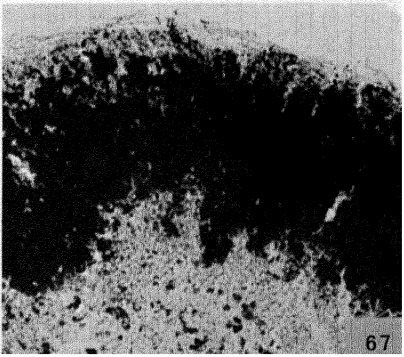
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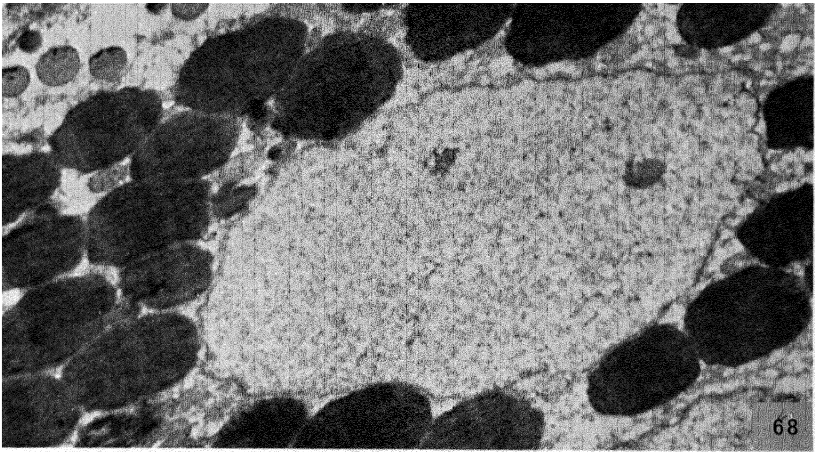
68. Electron micrograph of a portion of a "cortical" cell in the frog interrenal gland. Tissue fixed in 1 per cent buffered osmium tetroxide [134], embedded in n-butyl methacrylate, × 7,500. Courtesy Drs. M. H. Burgos and D. W. Fawcett. The lipide droplets (black) have remained essentially spherical, although slightly compressed during sectioning.

69. Electron micrograph of a portion of a hepatic cell in the frog liver. Tissue fixed and embedded as in Figure 68, × 12,000. Courtesy Dr. D. W. Fawcett. The lipide droplets, spherical in life, have assumed bizarre myelin forms.

70. Portion of a frog mesentery, mounted intact, fixed in formalin and stained with sudan black B, × 410. Courtesy Dr. D. W. Fawcett. The field is occupied by crowded white-adipose cells, which are compressed into polygonal forms. In some of the cells the lipide has crystallized (arrow); the crystals do not stain.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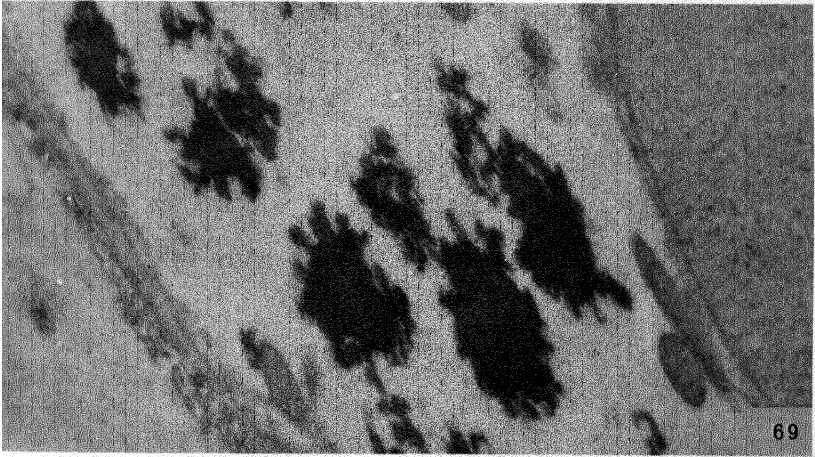
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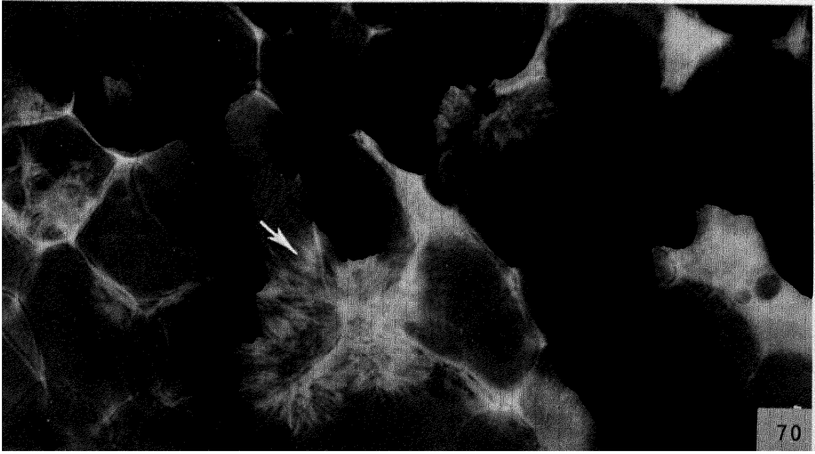
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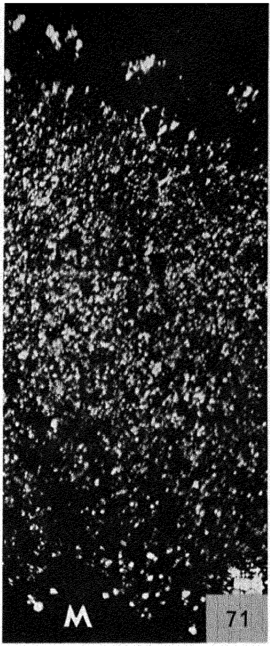
Steroid Reactions; Lipide Pigments

71. Adrenal cortex of a rat. Fixed in 10 per cent formalin for a week, sectioned on the freezing microtome, and mounted unstained in glycerol-gelatin; photographed under the polarizing microscope, × 100. Some of the lipides, mostly cholesterol esters, have crystallized during fixation. M, medulla.

72. Adrenal cortex of a rat from the control group in Table 3. Fixed in 10 per cent formalin for a week, sectioned on the freezing microtome, mounted with gelatin, oxidized for 3 days with 2.5 per cent iron alum and then treated with a 1:1 mixture of sulfuric and acetic acids (Schultz reaction), × 50. The lipide droplets throughout the cortex react fairly intensely. M, medulla.

73. Adrenal cortex from a rat in the experimental group in Table 3. Prepared as in Figure 72. The cortex is broader than normal, and the intensity of the cholesterol reaction in the lipides is greatly reduced. M, medulla.

74.–77. Ovaries from old mice in which lipide pigment ("brown degeneration") has been deposited in the interstitial cells [58]. The blocks were fixed in 10 per cent formalin, washed, sectioned on the freezing microtome, and then immersed in absolute acetone at room temperature. Figure 74, extracted for 24 hours, stained for 7 minutes with sudan black B, × 90; Figure 75, extracted for ½ hour, mounted unstained, and photographed under the fluorescence microscope, × 110; Figure 76, extracted for ½ hour, oxidized for 1 hour in 4 per cent chromic acid, and stained with the Schiff reagent, × 90; Figure 77, extracted for ½ hour and stained for cholesterol by the Schultz method, × 90.



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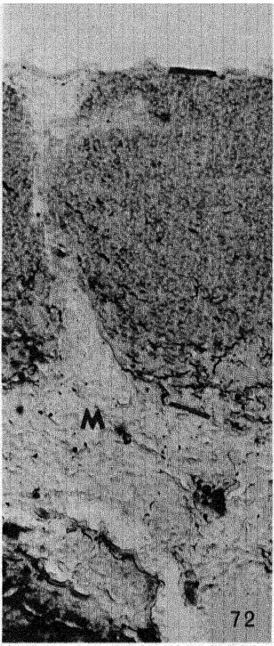

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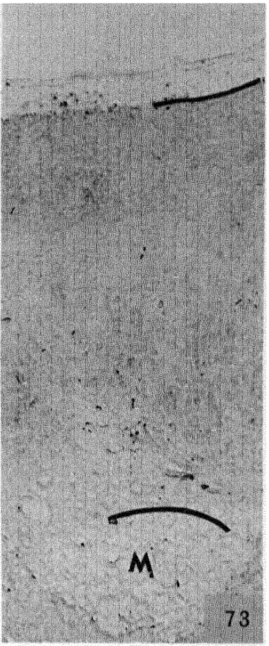

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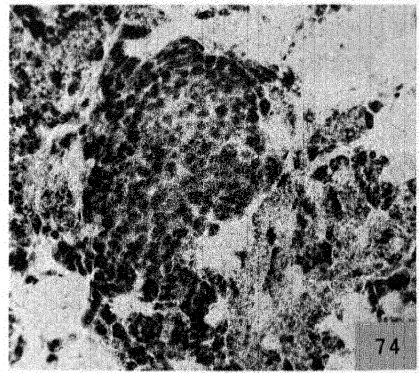
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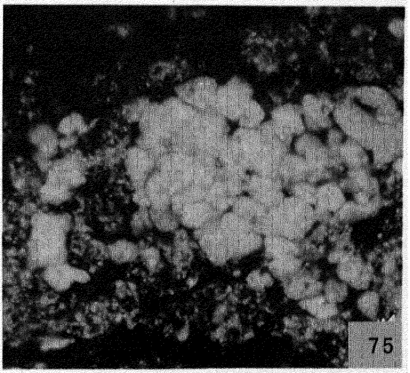
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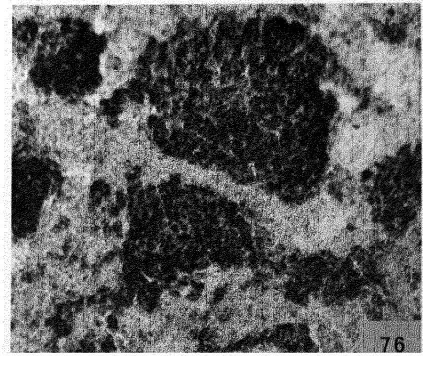
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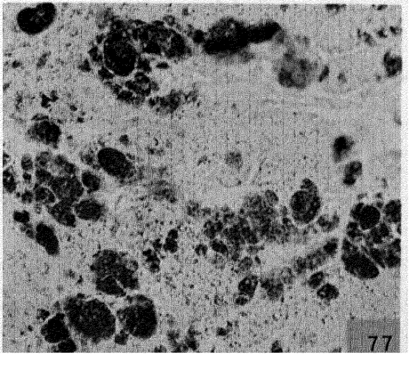
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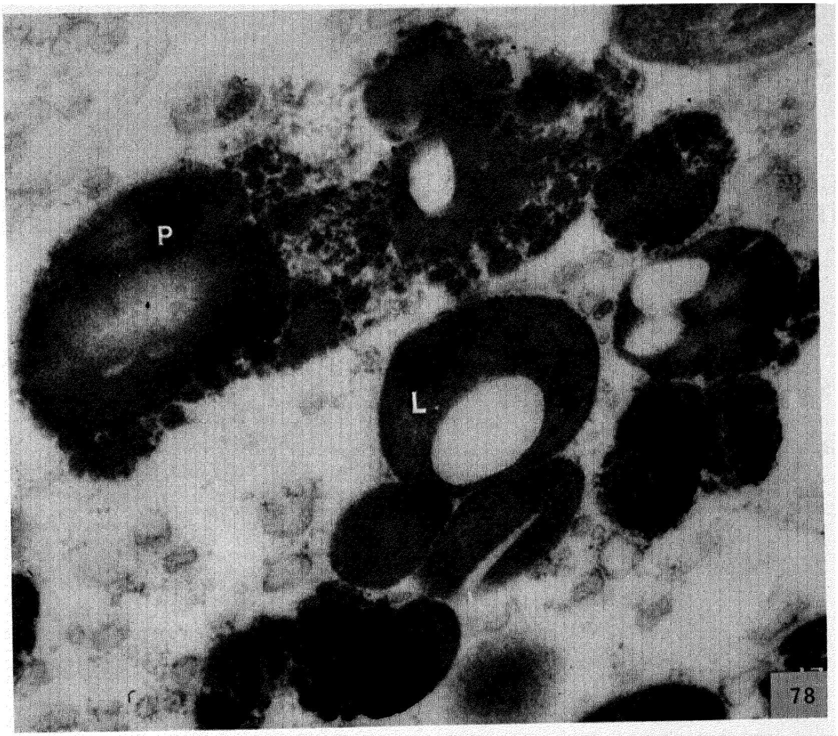
Lipide Pigments

78. Electronmicrograph of accumulations of lipide pigment (P) in a Leydig cell of the human testis. Fixed in 1 per cent buffered osmium tetroxide, × 44,000. Courtesy Drs. D. W. Fawcett and M. H. Burgos. L, lipide droplet; the significance of the central vacuole is obscure.

79. Electronmicrograph of an accumulation of lipide pigment above the nucleus of an epithelial cell of the human oviduct. Fixed in 1 per cent buffered osmium tetroxide, approximately × 12,000. Courtesy Dr. D. W. Fawcett. In this site, the occurrence of nonosmiophilic and presumably nonlipide droplets among the lipide pigment granules is apparent. P, pigment; D, droplet; M, mitochondrion.



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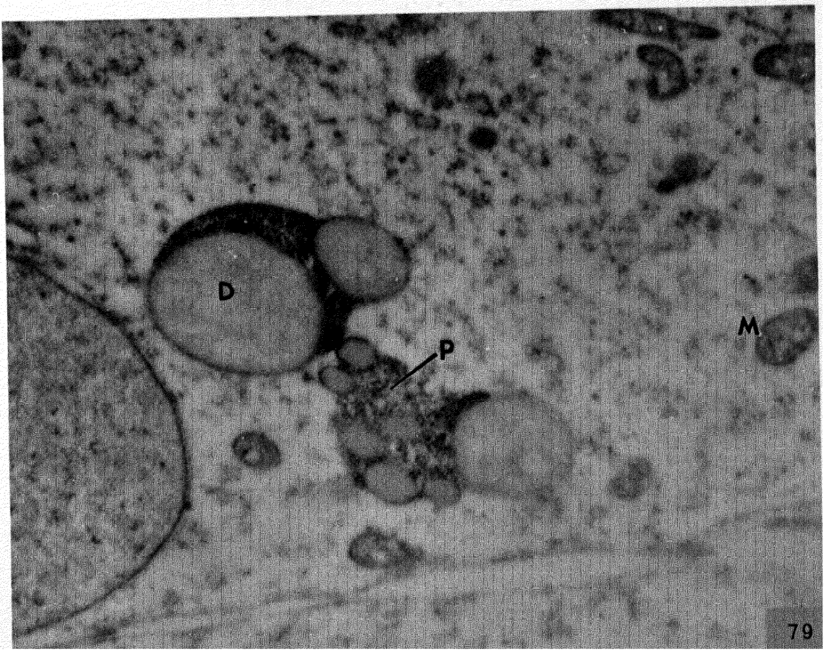


79

Adipose Tissues

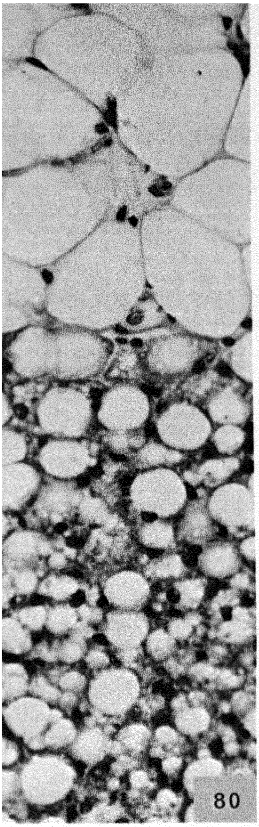
80. White-adipose tissue (above) and
rat. Fixed in 10 per cent formalin, sec
and eosin, × 300. Courtesy Dr. D. V
in size, shape, amount of cytoplasm a
the two types of adipose tissue.

81. Brown-adipose tissue from a rat. Fixed and stained according to Baker's [10] method for phosphatides, × 1,000. Courtesy Dr. D. W. Fawcett. The abundant mitochondria lying in the interdroplet cytoplasm are intensely stained, but the droplets themselves are negative for phosphatides.

82. White-adipose tissue (above) and brown-adipose tissue (below) from a rat that received 5 units of protamine zinc insulin 24 hours previously and was then allowed to eat *ad libitum*. Block fixed in alcohol-picric acid-formalin, sectioned in paraffin, and stained by the periodic acid-Schiff technique, × 160. Courtesy Dr. D. W. Fawcett. Glycogen (dark) occurs in traces in the white-adipose cells and abundantly in the brown-adipose cells.

83. Adrenal and peri-adrenal fat from a 9-day old rat. Fixed in 10 per cent formalin, sectioned at 25 μ on the freezing microtome, and stained with sudan black B, × 95.

84. Adrenal and peri-adrenal fat from a 2-day old rat. Fixed in 10 per cent formalin for a week, sectioned at 15 μ on the freezing microtome, and stained by the Ashbel and Seligman [6] hydrazide method for carbonyl groups, × 80. While virtually all of the lipide droplets in the adrenal display the pseudoplasmal reaction, the peri-adrenal fat shows reactivity in only occasional droplets.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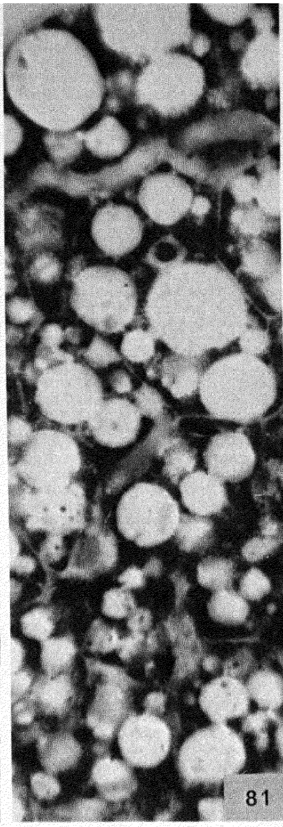
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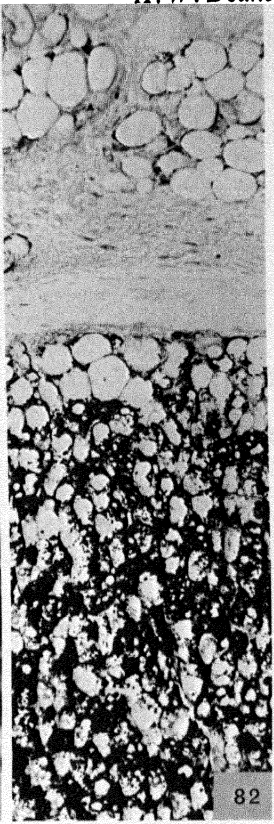
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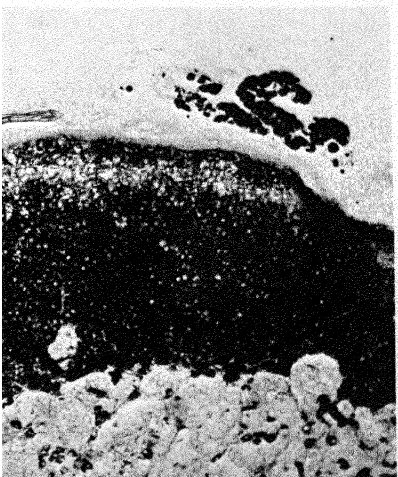
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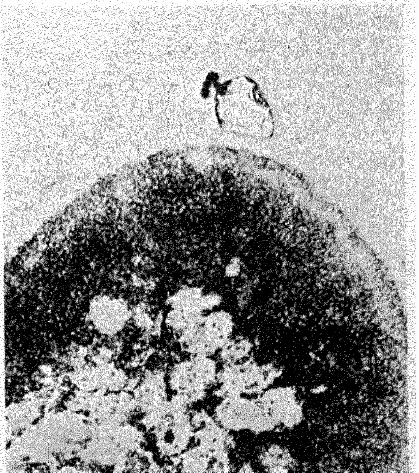
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Liver

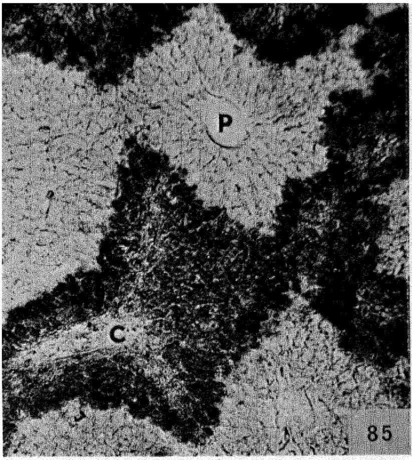
85. Liver of a mouse injected once daily for 4 days with 0.03 ml. chloroform, killed 24 hours following the last injection. Fixed in formalin, sectioned on the freezing microtome, and stained with sudan IV, × 60. Lipide is abundant in the hepatic cells lying in the central half of the lobule but virtually absent from those lying in the peripheral half. C, central vein; P, portal canal.

86. Liver of a rat that received for 4 weeks a highly purified diet deficient in biotin and inositol [60]. Fixed in formalin, sectioned on the freezing microtome, and stained with sudan IV, × 130. Small lipide droplets occur principally in the cells near the central vein (C); P, portal canal.

87. Liver of a rat that received the same purified diet for 5 weeks [60]. Prepared as in Figure 86. The lipide has increased in quantity, and in many cells the droplets have coalesced, thus filling the entire cytoplasm.

88. Same liver as Figure 86. Frozen section stained with the Schiff reagent. The lipide droplets display a moderate pseudoplasmal reaction.

89., 90. Liver of a 45-day old mouse. Fixed in 10 per cent formalin, postchromated, sectioned at 3 μ in paraffin, and stained by the long iron alum-hematoxylin procedure of Heidenhain [53], × 1,350. Figure 89 shows the cells adjacent to the portal canal which contain abundant, large, and intensely stained mitochondria. Figure 90 shows the cells adjacent to the central vein which possess sparser, smaller, and less intensely stained mitochondria.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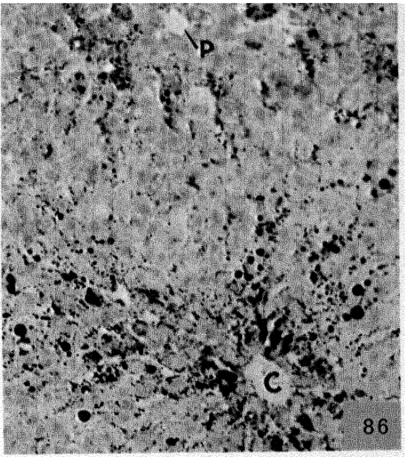
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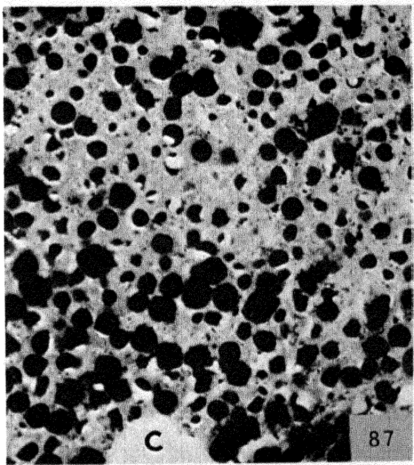
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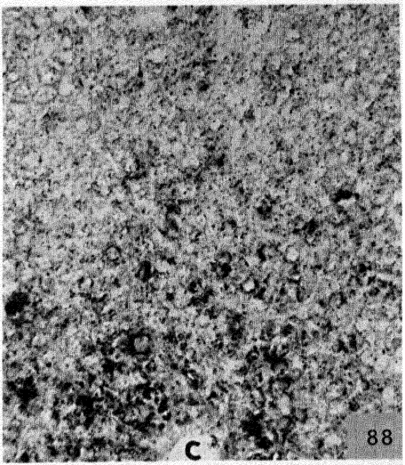
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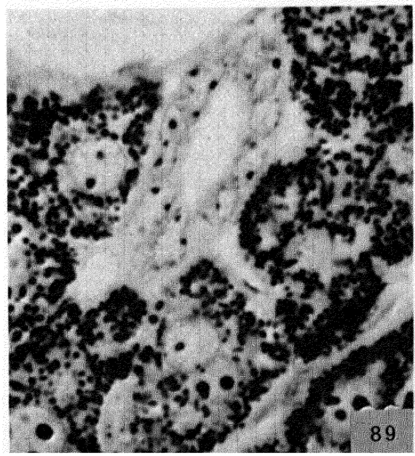
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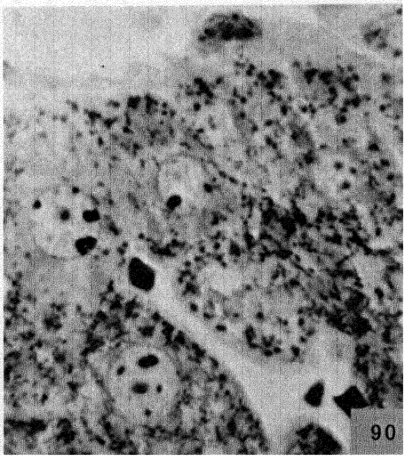

90

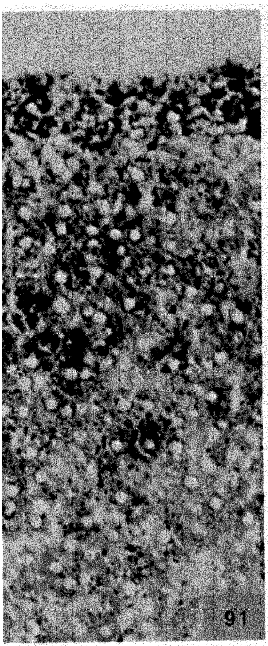
Adrenal Cortex

91., 92. Adrenal cortex of a rat. Sectioned unfixed at 10 μ in the cryostat [9], fixed briefly in 10 per cent formalin, × 200. Figure 91, stained for 7 minutes with sudan black B; Figure 92, stained for cholesterol by the Schultz method. Only a fraction of the lipide droplets that stain with sudan, generally the larger ones, contain detectable amounts of cholesterol.

93. Adrenal cortex of a female hamster. Fixed in 10 per cent formalin for a week, sectioned at 15 μ on the freezing microtome, and stained for 7 minutes with sudan black B, × 300. Although there are occasional small lipide droplets in the cells of the zona glomerulosa (G), there are none in the cells of the zona fasciculata (F).

94., 95. Adrenal cortex of a female rat, hypophysectomized 3 weeks earlier (Table 4) [105]. Fixed for a week in 10 per cent neutral buffered formalin, washed, and sectioned on the freezing microtome at 15 μ. Figure 94, stained for 1 minute with sudan black B; Figure 95, stained for 30 minutes in the Schiff reagent; both × 50. Some of the lipides present in the inner fasciculata fail to give the Schiff reaction (in the adrenal of the intact rat the lipides throughout the cortex react). G, glomerulosa; F, fasciculata.

96., 97. Adrenal cortex of a female rat, hypophysectomized 6 weeks earlier (Table 4) [105]. Prepared as in Figures 94 and 95 respectively. None of the lipides persisting in the fasciculata reacts to the pseudoplasmal test. G, glomerulosa; F, fasciculata.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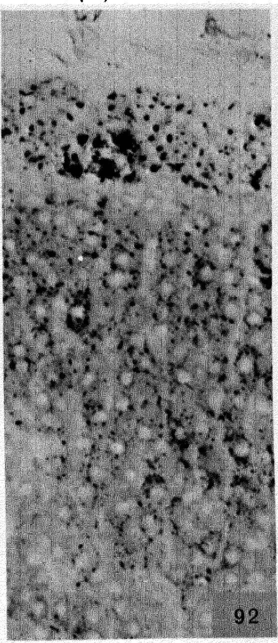
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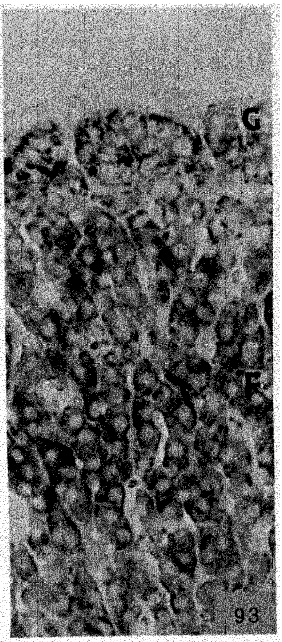

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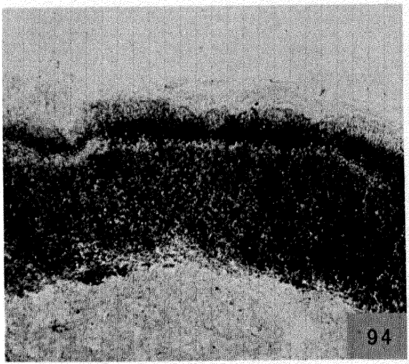
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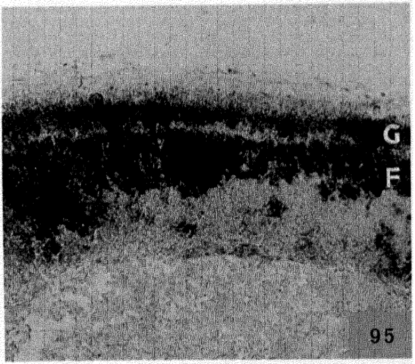

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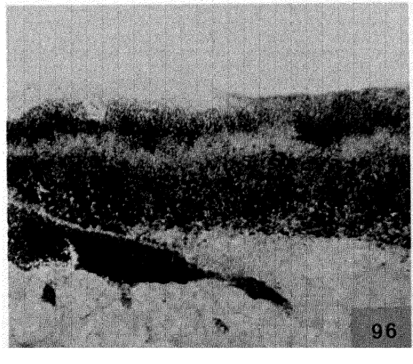
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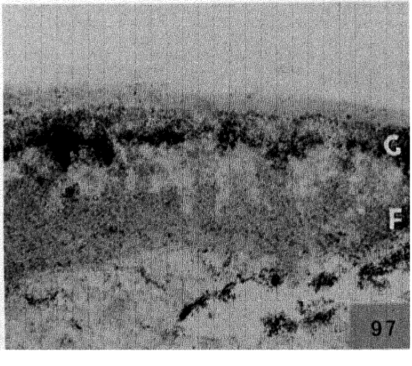


97

Photomicrographs of 10 μ sections of tissue fixed in 80 per cent ethyl alcohol and 1 per cent trichloracetic acid (80-TCA) except that in Figure 101, which was fixed in Zenker's fluid. All sections were stained with the DDD method for sulfhydryls except Figure 102, which was stained for both sulfhydryls and disulfides.

98. Rat liver. Hepatic cells stain uniformly for sulfhydryls throughout the liver lobules. × 100.

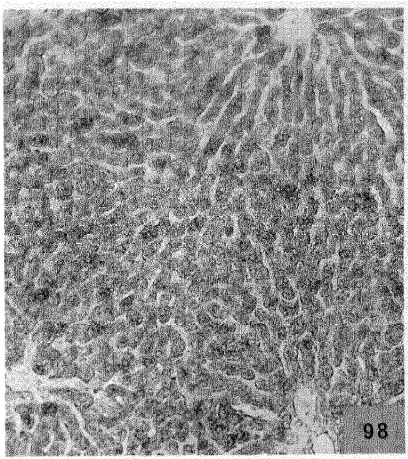
99. Rat kidney cortex. The renal tubular epithelium is positive for sulfhydryls, but the brush border of the proximal convoluted tubules is unstained. Vascular endothelium and the epithelium of the glomeruli are weakly stained. × 75.

100. Rat testis. Cells of the seminiferous tubules are moderately positive and interstitial cells are somewhat more intensely reactive, whereas the sperm react strongly for sulfhydryls. × 75.

101. Monkey retina. Distal rod segment stains most intensely for sulfhydryls at the top of the photomicrograph. The dark line below the rods is an intense reaction at the external limiting membrane. × 300.

102. The brush border of the proximal convoluted tubules of the rat kidney is reactive for both sulfhydryls and disulfides after this fixation (cf. Fig. 99). × 200.

103. Rat epididymis. Cytoplasm of the epithelium of the epididymis is positive for sulfhydryls. Spermatozoa in the centers of the tubules are especially reactive. × 200.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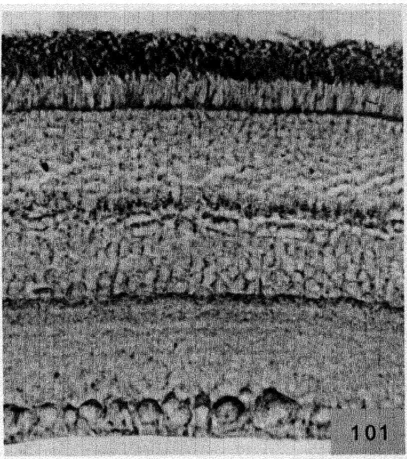
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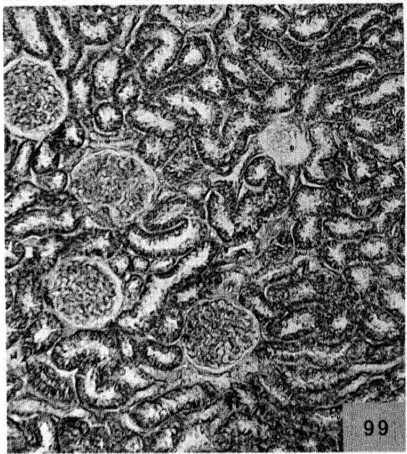
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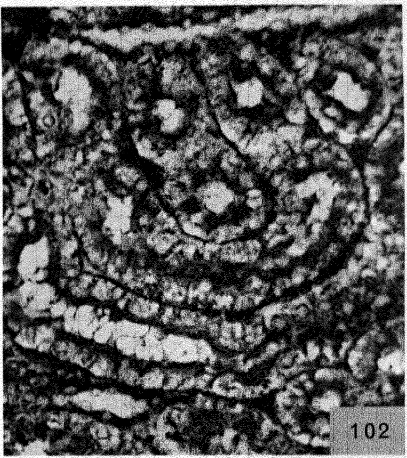
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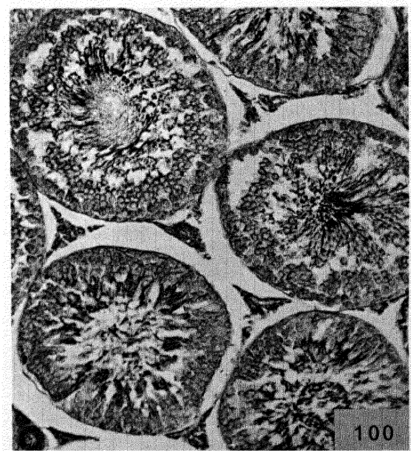
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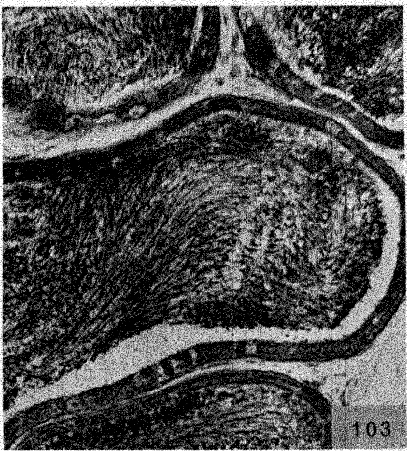

103

Figures 104 and 105 are of 10 μ sections of tissue fixed in 80 per cent ethyl alcohol and 1 per cent trichloracetic acid (80-TCA) and the remainder were from sections of blocks fixed in Zenker's fluid. All are stained for both sulfhydryls and disulfides; the first two with the tetrazolium method, the remaining with the DDD method after reduction of the disulfides.

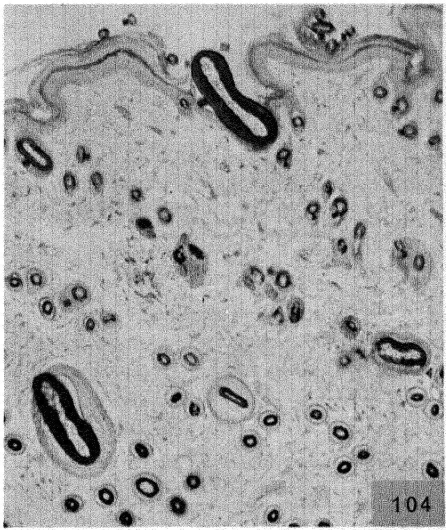
104. Rat skin. The epidermis is moderately reactive and contains a band of intensely reactive material just distal to the stratum Malpighii. This intensely staining band is not always present in normal skin but occurs regularly toward the end of the hair cycle. In the dermis the collagen bundles are unstained, but connective tissue cells stain weakly. Numerous cross sections of hairs are present, and the root sheaths are weakly stained, while the hair cortices are intensely reactive. This region of the cortex of the hair shaft is negative for sulfhydryl, indicating that the staining is due only to disulfides. × 50.

105. Same section of tissue as Figure 104, showing deeper parts of dermis. At bottom of photomicrograph, skeletal muscle is moderately reactive. Cross sections through the hairs indicate a rapidly intensifying reaction in the hair cortex from bulb to the region of horny transformation, indicating increased numbers of reactive groups. Most of the staining in these cross sections of the hair cortex is due to sulfhydryl groups only. × 50.

106. Rat aorta. A positive reaction for sulfhydryl and disulfide is obtained in the bands of elastic tissue, whereas the same structures are negative for sulfhydryl alone after 80-TCA fixation. Smooth muscle fibers between the elastic bands are less reactive. × 200.

107. Rat stomach. The cytoplasm of parietal cells stains intensely after this fixation but not after 80-TCA fixation, indicating that protein, rich in sulfhydryls and disulfides, is retained in these cells by the fixative used. The surface epithelium stains strongly also. × 100.

108. Rat trachea. A globular material is retained by this fixation, which is intensely reactive for sulfhydryls and disulfides in the apices of the tracheal epithelial cells. Subjacent to the epithelium, elastic tissue and reticular fibers are stained. The epithelium of the tracheal glands and ducts are moderately positive. × 350.



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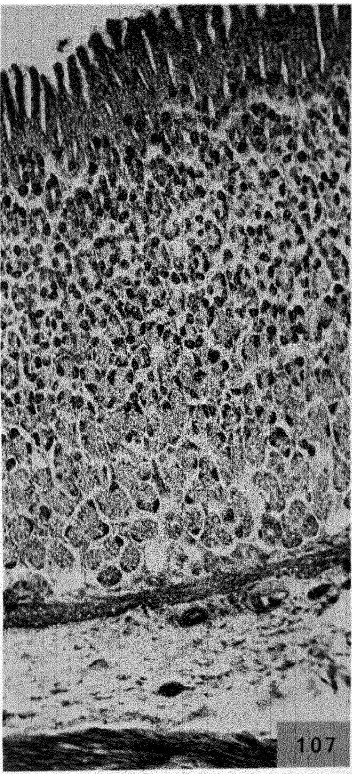

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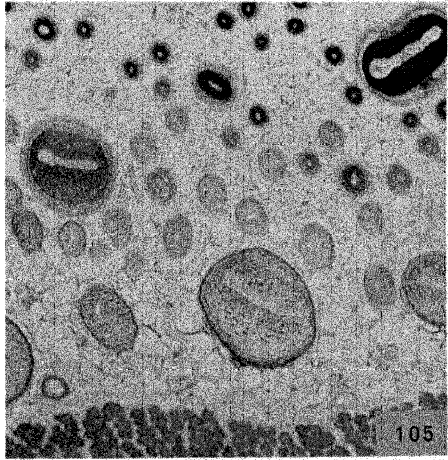

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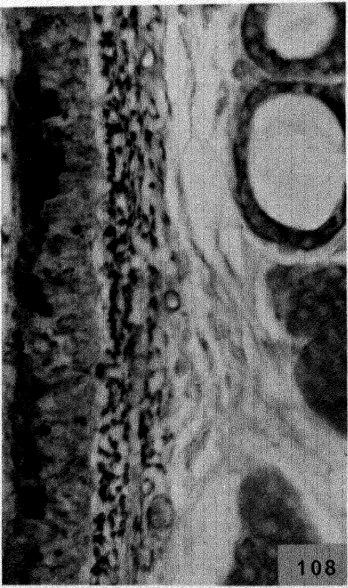

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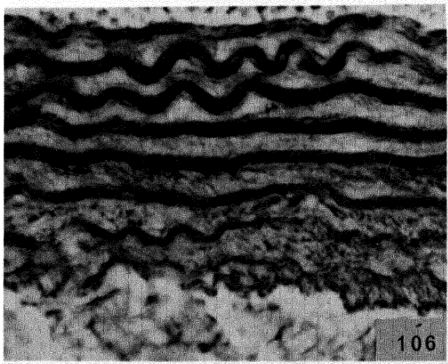


106

All photomicrographs are from sections of rat pancreas which were stained for sulfhydryls and disulfides with the DDD method.

109. 80 per cent ethyl alcohol and 1 per cent trichloracetic acid fixation. The acinar cells stain moderately, especially in their basal parts, whereas the islet cells stain weakly and the cell types cannot be differentiated on the basis of the staining reaction. × 100.

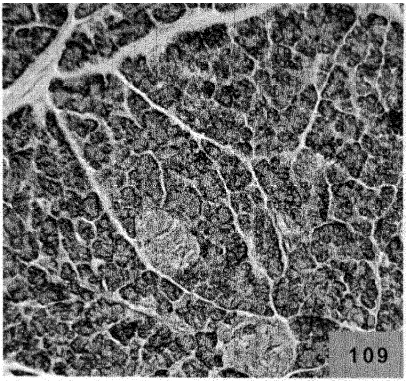
110. Zenker's fixation. In contrast to Figure 109, a material is retained in the apices of the pancreatic acinar cells which is intensely reactive. This material is not present in the pancreases of animals which had been injected with pilocarpine regardless of the fixative used, and therefore it can be equated with zymogen material which contains enzymes rich in sulfhydryls and disulfides. × 350.

111. Romeis' fixation. Islet of normal rat. After this fixation, insulin is retained *in situ* and the beta cells of the islets of Langerhans stain intensely. Compare with islets in Figure 109, in which insulin was dissolved during fixation. Smaller, peripherally placed, alpha cells (top of islet) are weakly stained. Acinar cells are also strongly reactive. × 440.

112. Romeis' fixation. Islet of rat injected with high doses (2.5 mg/day) of cortisone for 12 days. There is an obvious decrease in the content of sulfhydryl and disulfide-positive material in the beta cells in comparison to the normal control (Fig. 111). Acinar cells stain similarly to those in the control pancreas. × 440.

113. Romeis' fixation. Islet of the rat injected with low doses ($50\mu g$/day) of cortisone for 24 days. There is a slight decrease in the sulfhydryl and disulfide-positive material of the beta cells in comparison to the normal control (cf. Fig. 111). Staining reaction of acinar cells was unaffected. × 440.

114. Romeis' fixation. Islet of rat which had been adrenalectomized for 24 days with no replacement therapy other than saline drinking water. This condition caused a slight decrease in the sulfhydryl and disulfide-positive material in some of the islet cells. × 440.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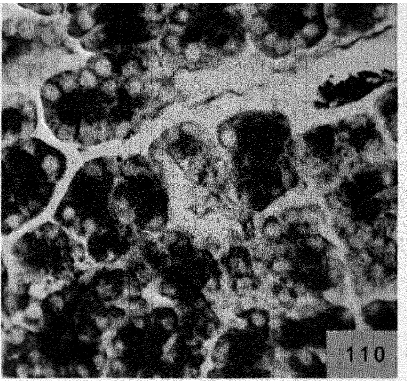
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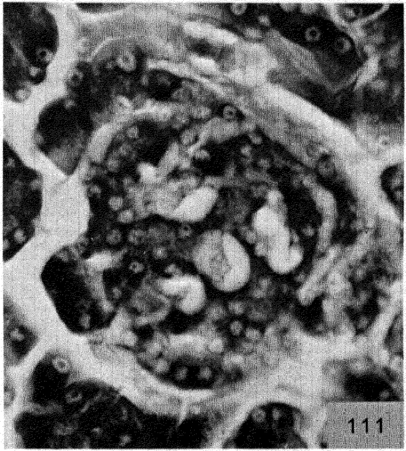
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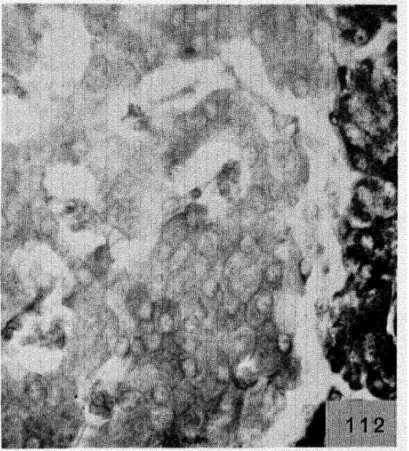
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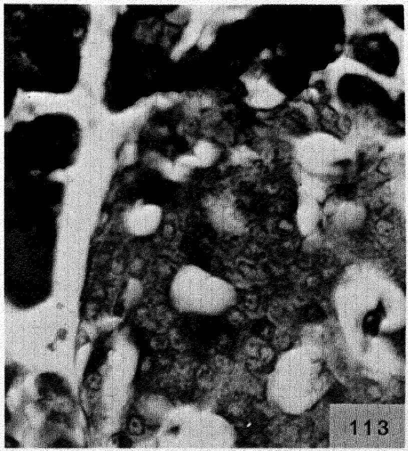
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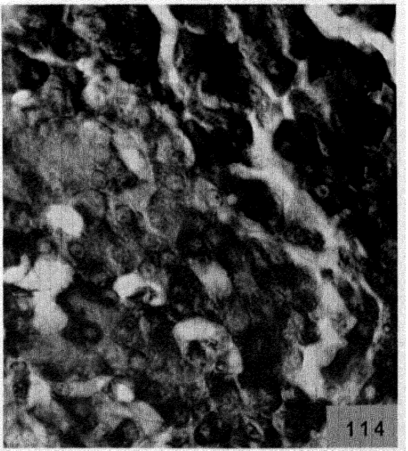
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All photomicrographs are taken from sections of rat pituitary gland stained for sulfhydryls and disulfides. All glands were fixed in sublimate-formol, except that in Figure 115 which was fixed in 80 per cent ethyl alcohol and 1 per cent trichloracetic acid.

115. Section of rat pituitary gland showing anterior lobe (bottom), intermediate lobe (upper center) and neural lobe (top). In the anterior lobe, the cells stain with uniform intensity (cf. Fig. 119) and in the neural lobe the nerve fibers stain weakly, but no neurosecretory material is retained by this fixative (cf. Fig. 116). × 125.

116. Neural lobe of normal rat. Numerous globules and vesicular accumulations of neurosecretory material are retained and are intensely reactive. × 350.

117. Neural lobe of rat dehydrated for 7 days. Neurosecretory material has been completely depleted. Erythrocytes in the blood vessels are reactive for sulfhydryls and disulfides. × 600.

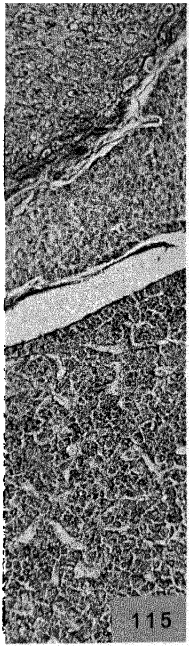
118. Neural lobe of rat 7 days after section of the infundibular stalk. Neurosecretory material is absent and the staining for sulfhydryl and disulfide groups is less than that seen in the control sections. × 600.

119. Anterior lobe of normal rat. Only the acidophils are intensely reactive for sulfhydryls and disulfides after sublimate-formol fixation. Larger, weakly staining basophiles can be seen in the center of the photomicrograph. × 175.

120. Anterior lobe of rat, 5 minutes after a painful stress. The amount of disulfide-positive material in the acidophiles is decreased (cf. Fig. 119). × 175.

121. Anterior lobe of rat fed propylthiouracil for 40 days. The amount of sulfhydryl and disulfide-positive material has been decreased in all cells, especially acidophiles, so that the entire gland is unreactive. × 175.

122. Anterior lobe of rat which had been castrated 90 days previously. Acidophiles are stained as in the normal. Large basophiles containing colloid-filled vacuoles are present. Although the colloid is negative for sulfhydryls and disulfides, the granular cytoplasm is moderately reactive. × 550.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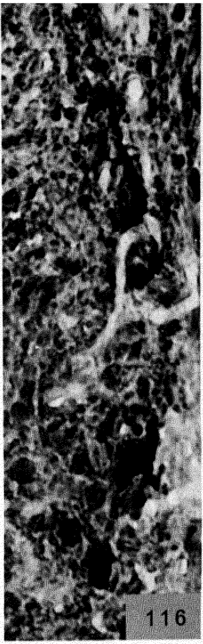
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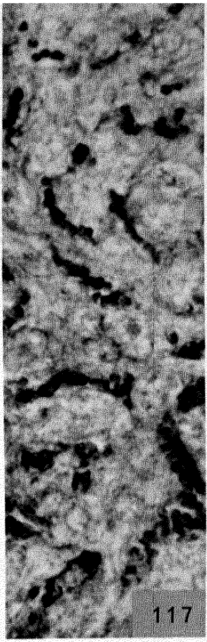
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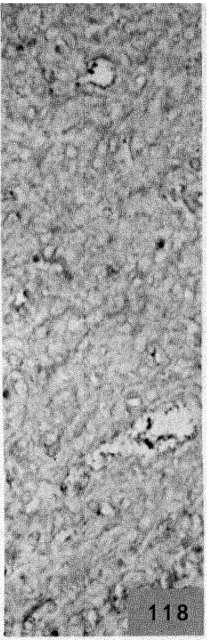
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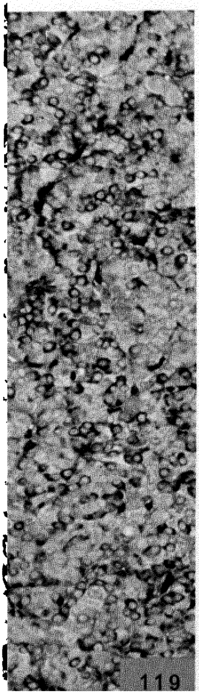
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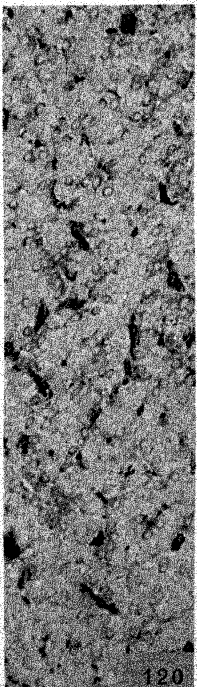
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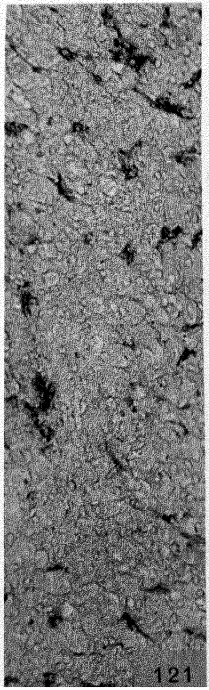
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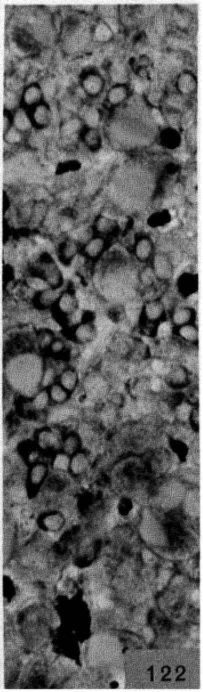

122

All photomicrographs illustrate histological sections stained by the PAS method of anterior pituitary glands of normal male rats both before and after various extraction procedures.

123. The "sex zone" of the anterior pituitary fixed in sublimate-formol, showing the distribution of PAS-positive cells in this region. Intermediate lobe at bottom. × 115.

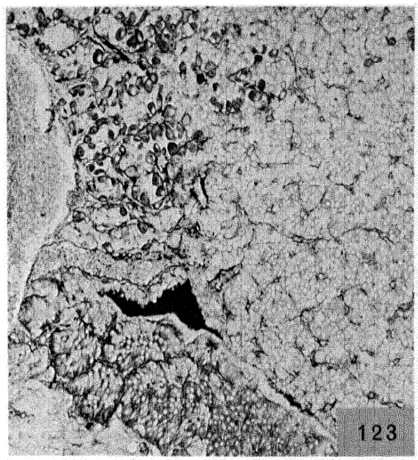
124. A part of the "sex zone" of a gland, extracted with Na_2SO_4 at pH 4.4 for 24 hours and refixed in sublimate-formol. A number of heavily staining cells is seen, although there appear to be fewer than in either Figure 123 or Figure 125. Bioassay of glands extracted with Na_2SO_4 revealed a decrease in the amount of FSH, but a normal amount of LH and TSH. Intermediate lobe in lower left. × 115.

125. A part of the "sex zone" of a gland which was placed in absolute acetone for 24 hours and then refixed in sublimate-formol before further histological processing. A large number of shrunken, heavily staining, PAS-positive cells are present (cf. Figs. 123, 126). The experiment indicates the feasibility of using acetone for the fixation of pituitaries for bioassay and comparing these results with those obtained from morphological study of pituitaries fixed in sublimate-formol. × 115.

126. A similar area of a gland as that in Figure 125, which was fixed in absolute acetone for 24 hours, but here, without refixation in sublimate-formol. Although the cells are intact, there appears to be a general loss of stainable cytoplasm, especially glycoprotein. This indicates the reversibility of the denaturation of protein fixed in acetone by introduction of sections into water. × 115.

127. Section of anterior pituitary fixed in dilute ammonium hydroxide (pH 8) and refixed in sublimate-formol. All hormones are extracted with ammonium hydroxide and histologically there is a complete absence of cells and cell contents. What remains is a demonstration of the reticulum of the gland, which is PAS-positive. × 115.

128. Section of anterior pituitary extracted with physiological saline for 24 hours and refixed in sublimate-formol. Saline is a nonspecific solvent in that it extracts a variable amount of the glycoprotein hormones but none completely. Some residual PAS-positive material remains in the basophiles, and the acidophiles are also partially extracted. Nuclei counterstained with hematoxylin. × 800.



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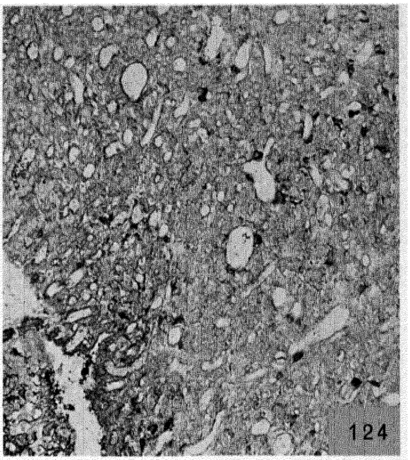

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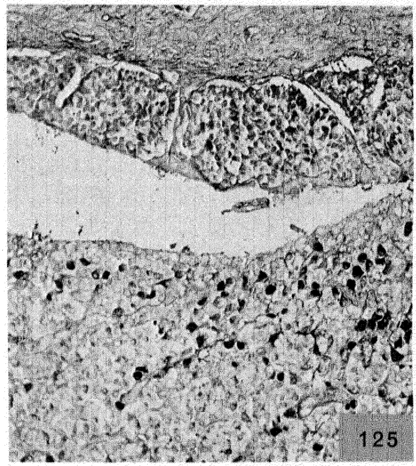

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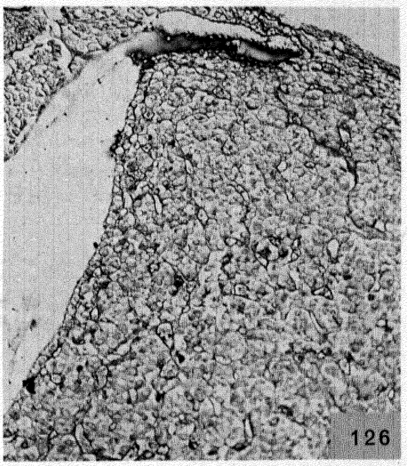

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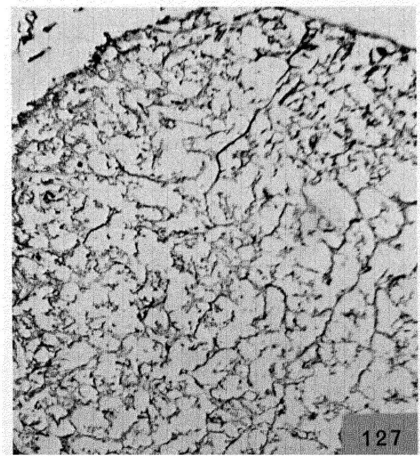

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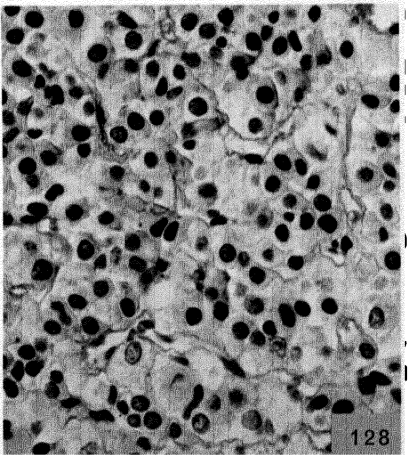


128

All photomicrographs are taken of part of the "sex zone" of rat anterior lobe stained with the PAS method. In Figures 130, 133, and 134, the nuclei were counterstained with hematoxylin. All figures × 800.

129. Sublimate-formol fixation. In the normal rat, two types of PAS-positive cells are seen. One is more coarsely granulated and darker-staining; the other is finer granulated and lighter-staining.

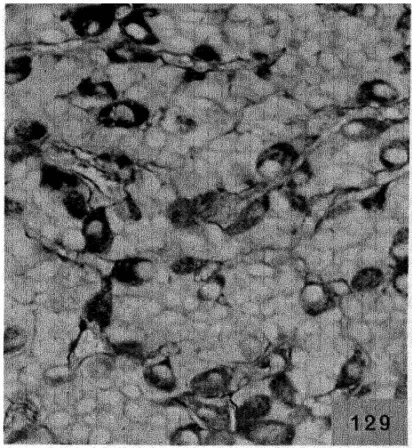
130. Sublimate-formol fixation. Section of pituitary 90 days after castration, showing the typical and well-known changes; increased number of PAS-positive cells containing increased amounts of glycoprotein and a "signet ring" vacuole filled with unreactive colloid (cf. Fig. 129).

131. Normal gland extracted with 2.5 per cent trichloracetic acid and refixed in sublimate-formol. This procedure extracts FSH and TSH completely but leaves LH. There is little or no PAS-positive material left in these cells. What glycoprotein is present appears dustlike and does not stain intensely.

132. Pituitary gland 90 days after castration, extracted with 2.5 per cent trichloracetic acid and refixed in sublimate-formol. Since the amount of LH increases following castration, and since it is the only glycoprotein remaining after this extraction, the accumulation of PAS-positive granules represents a more obvious histochemical demonstration of LH than in the normal.

133. Normal pituitary extracted with 50 per cent pyridine and refixed in sublimate-formol. This extraction removes all glycoprotein and causes swelling and absence of cytoplasmic contents of what were presumably PAS-positive cells.

134. Effect of 50 per cent pyridine extraction on pituitary of rat castrated for 90 days. Compare with Figures 130 and 133.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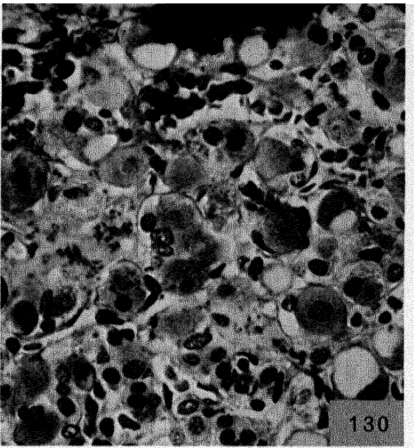
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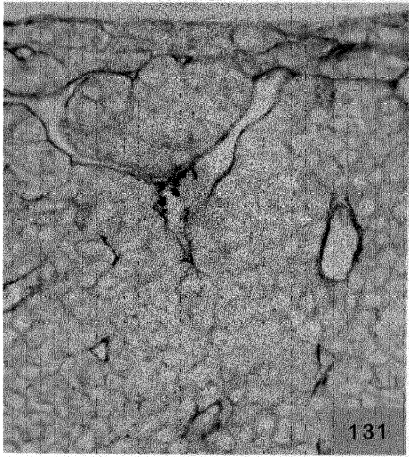
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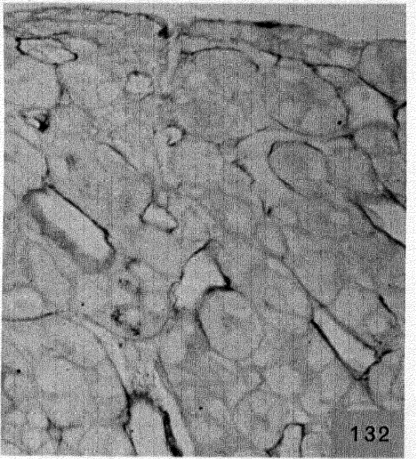
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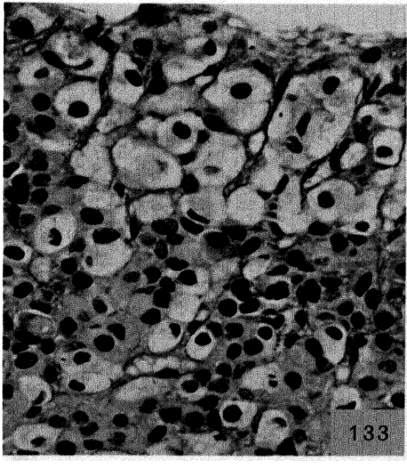
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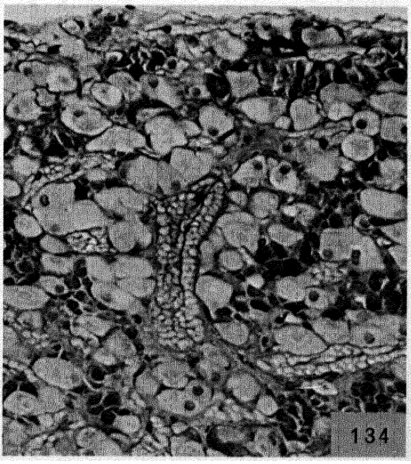

134

135. The electron micrograph shows a relatively large field in the basal region of a pancreatic acinar cell (guinea pig) and illustrates the two structurally distinct components found in typical ergastoplasm.

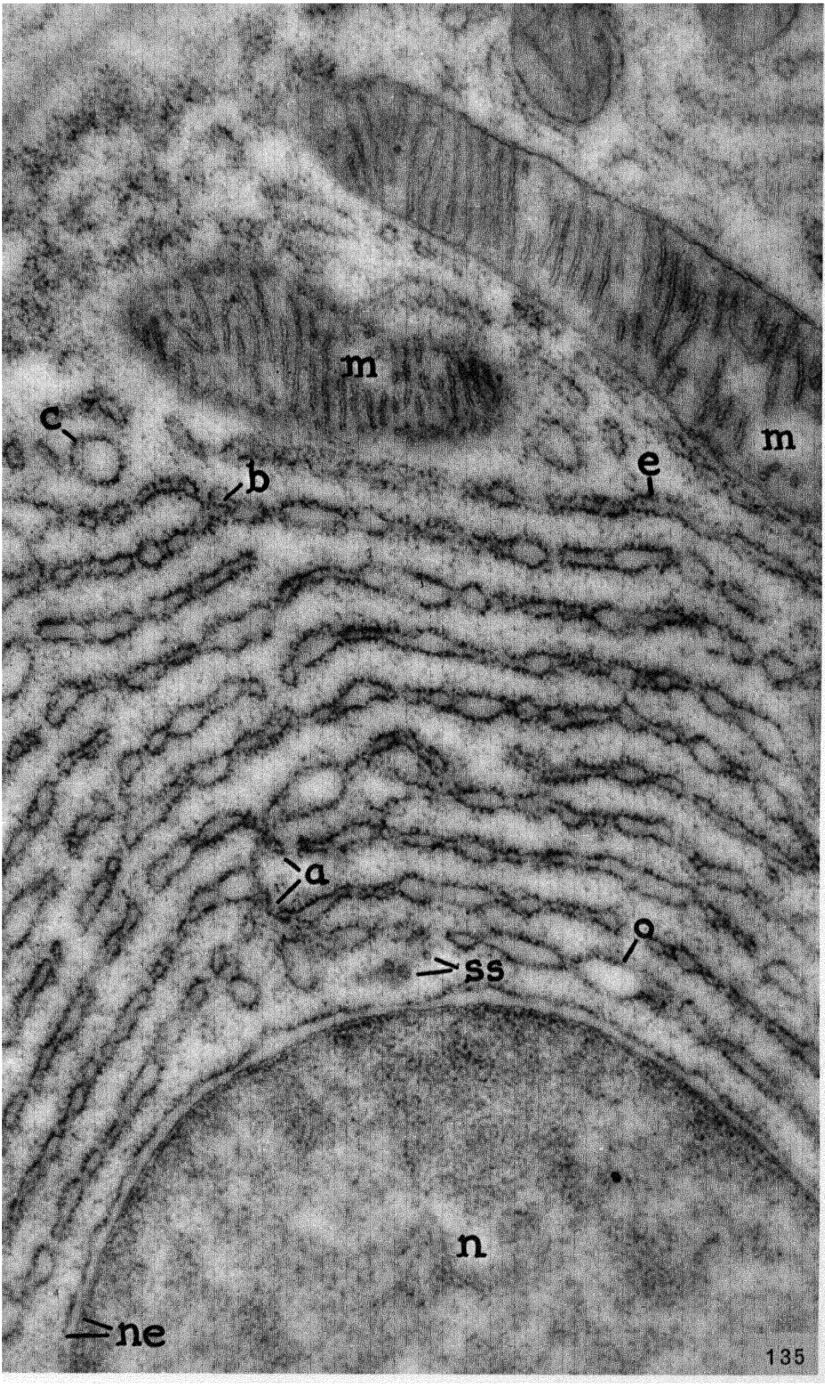
One component is represented by a voluminous and preferentially oriented endoplasmic reticulum (ER). Its membrane-bound elements appear in this section as rows of circular (*c*), oval (*o*), and elongated (*e*) profiles which are disposed parallel to one another at more or less regular intervals. In three dimensions each row corresponds to a fenestrated cisterna or to a reticular sheet. The regularity of the arrangement is disturbed in a few places by branching rows (*b*) and by anastomoses (*a*) between adjacent rows. Such connections ensure the continuity of the system throughout the cytoplasm. In this particular cell, the entire array of ER profiles is concentric with the nucleus (*n*).

The other component of the ergastoplasm is represented by numerous small, dense particles which are found either attached to the limiting membrane of the endoplasmic reticulum, or scattered freely in the intervening cytoplasmic matrix.

Most of the ER elements bear attached particles on the outer surface of their limiting membrane and as such appear rough-surfaced. Smooth-surfaced elements of the endoplasmic reticulum are rare in acinar cells and usually occur in small clusters (*ss*).

Note that the membranes of mitochondrial profiles (*m*) are free of attached particles, whereas the outer membrane of the nuclear envelope (*ne*) bears attached particles, like the limiting membrane of the endoplasmic reticulum. × 36,000.

This figure has been published in reference [26].



135

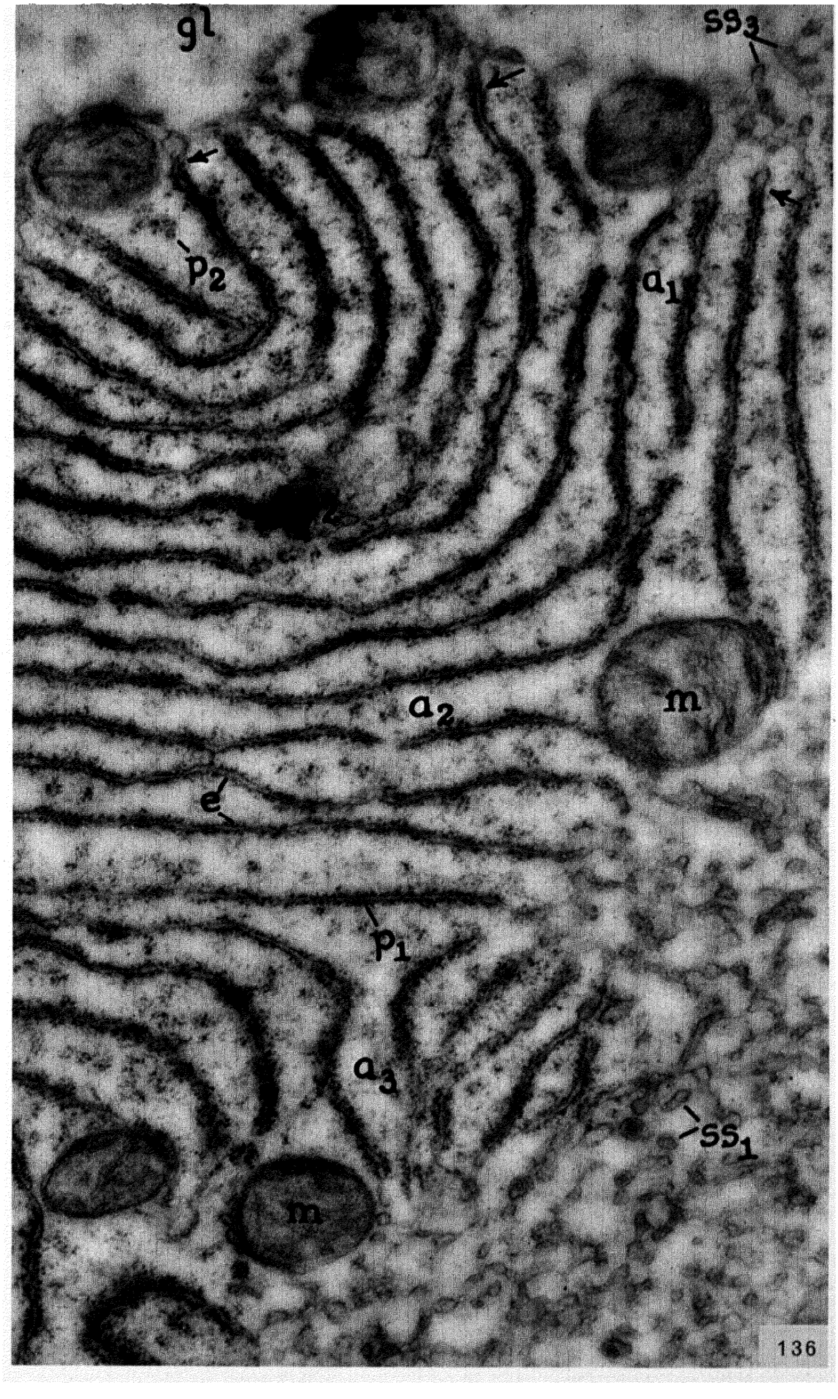
136. The micrograph illustrates the disposition of the endoplasmic reticulum and of the particulate component in the cytoplasm of parenchymal liver cells (rat). In this relatively large field, numerous elongated (*e*) profiles, bearing attached particles, are disposed parallel to one another at more or less regular intervals. They form three interconnected arrays (a_1, a_2, a_3). In a_1 and a_3 the profiles are vertically oriented, whereas in a_2 they are horizontally disposed. In three dimensions the elongated profiles of these arrays correspond to relatively large cisternae.

Smooth-surfaced elements of the endoplasmic reticulum are much more numerous than in pancreatic acinar cells and occur in distinct masses (ss_1, ss_2, ss_3), within which they form a tightly meshed, randomly oriented network. Numerous connections between the rough- and smooth-surfaced elements of the endoplasmic reticulum can be seen in the figure. Some of them are indicated by arrows.

Most of the small dense particles present in the cytoplasm are attached (p_1) to the membrane of preferentially oriented cisternae. A few free particles (p_2) are scattered individually, in chains, or in small clusters in the intervening cytoplasmic matrix. No particles occur on, or in between, the smooth-surfaced, randomly oriented part of the endoplasmic reticulum.

Mitochondrial profiles and the margin of a glycogen deposit can be seen at *m* and *gl* respectively.

In this cell type, as in many nerve cells, the masses of basophilic cytoplasm are as a rule discontinuous, and so are the masses formed by the rough-surfaced, preferentially oriented elements of the endoplasmic reticulum. × 38,000.


gl
ss3
p2
a1
a2
m
e
p1
a3
ss1
m
136

137. Relatively large field in the cytoplasm of an endothelial cell from a blood capillary (myocardium of adult rat).

The section cuts laterally through the capillary wall, and misses the lumen in this field. The cell membrane (cm_1, cm_2) shows numerous small invaginations and associated vesicles. At cm_1 as well as at cm_2 it faces the pericapillary spaces and appears coated by a more or less continuous layer of basement membrane material (*bm*).

The cytoplasm contains a mitochondrial profile (*m*), a compound vesicle (*v*) (cf. [31, 38]), and a scattering of profiles ascribable to the endoplasmic reticulum. Most of them are smooth-surfaced (*ss*) and only a few are partly covered with attached particles (*rs*).

A few particles appear scattered individually or in clusters (*p*) throughout the cytoplasmic matrix.

The margins of two adjacent heart muscle fibres can be seen at *mf*. × 40,000.

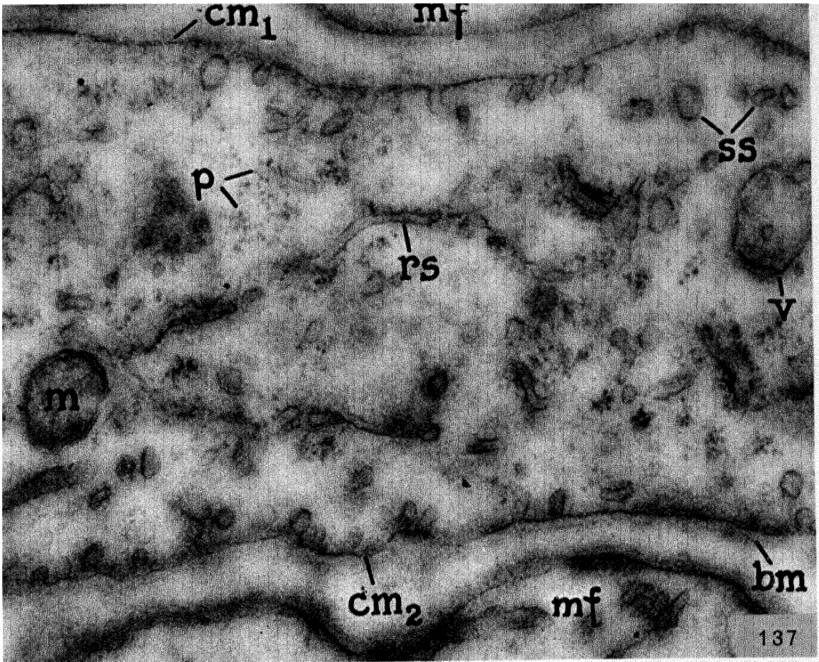
138. Large field in the cytoplasm of an endothelial cell from a blood capillary (myocardium of rabbit embryo).

The cell membrane appears at cm_1 and cm_2 facing, respectively, the lumen and the pericapillary spaces. As in Figure 137, small invaginations and associated vesicles can be seen along the sectioned cell membrane.

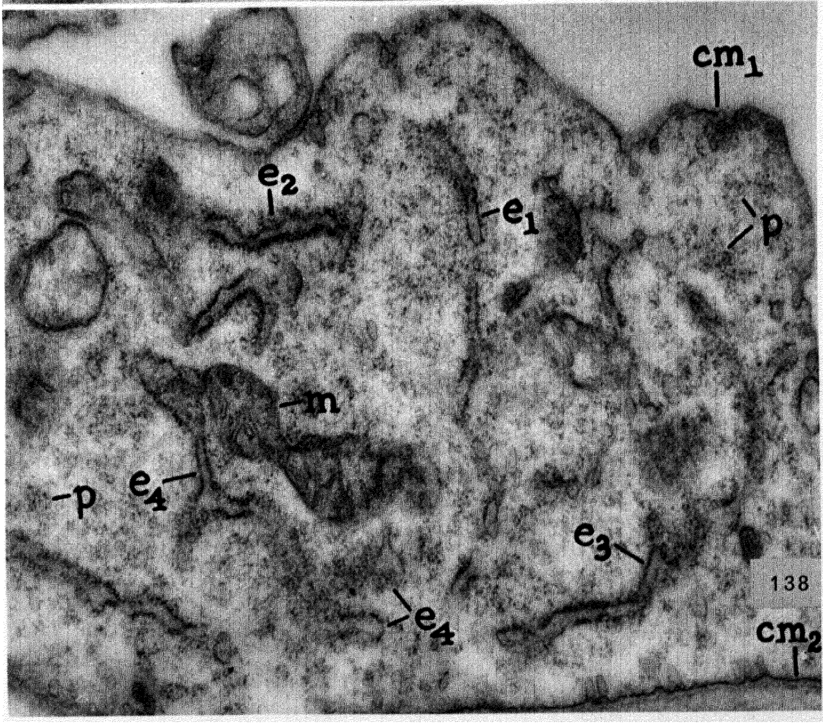
The cytoplasm contains a few mitochondria and relatively numerous elongated profiles of the endoplasmic reticulum. The latter correspond to normally (e_1) or obliquely (e_2, e_3, e_4) sectioned cisternae, are randomly disposed, and appear heavily coated with attached particles. In addition, numerous particles (*p*) can be seen freely and more or less evenly distributed throughout the entire cytoplasmic matrix.

The endothelial cells in the embryo are characterized by intense cytoplasmic basophilia.

A comparison with Figure 137 shows that embryonic endothelial cells contain an appreciably larger population of small dense particles than adult cells of the same type. The same obtains when the embryonic and adult cells belong to the same animal species. × 30,000.



137



138

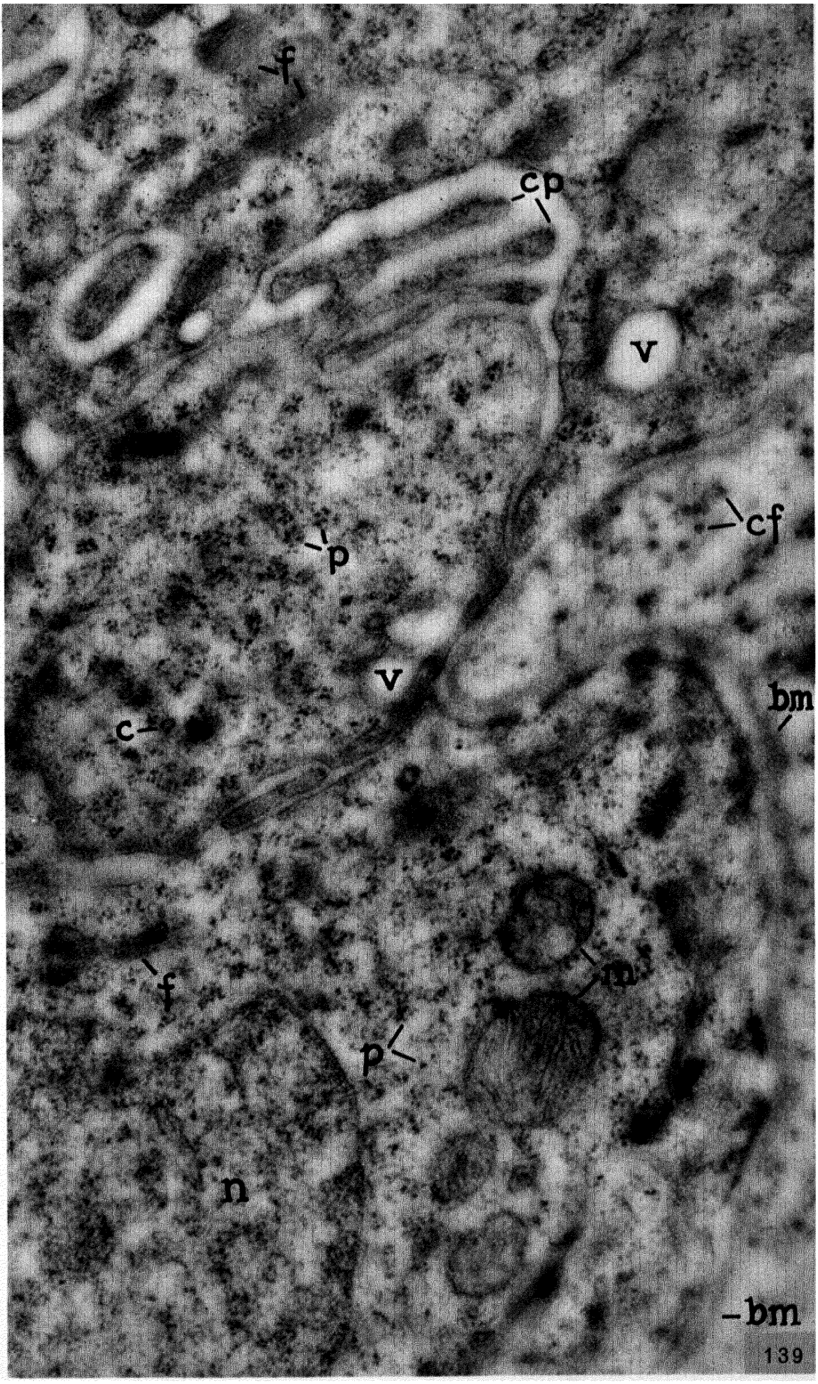
139. The micrograph shows parts of three cells in the *stratum germinativum* of lingual mucosa (rat).

The basement membrane is marked *bm* and collagen fibrils in the corium, *cf.*

The cytoplasm of the epithelial cells contains very few circular, smooth-surfaced profiles (*c*) ascribable to the endoplasmic reticulum. Large numbers of small dense particles (*p*) appear freely scattered throughout the cytoplasm either individually or in clusters. Only a narrow cortical layer and the laminated cell processes (*cp*) it forms are found free of small particles. The large "vacuoles" marked *v* probably represent sections through intercellular spaces.

Part of the nucleus (*n*) of one cell, mitochondrial profiles (*m*), and bundles of dense fibrillar material (*f*) (keratin?) can also be seen in the cytoplasm of the epithelial cells.

Cells of this type are also characterized by intense and diffuse cytoplasmic basophilia. × 44,000.

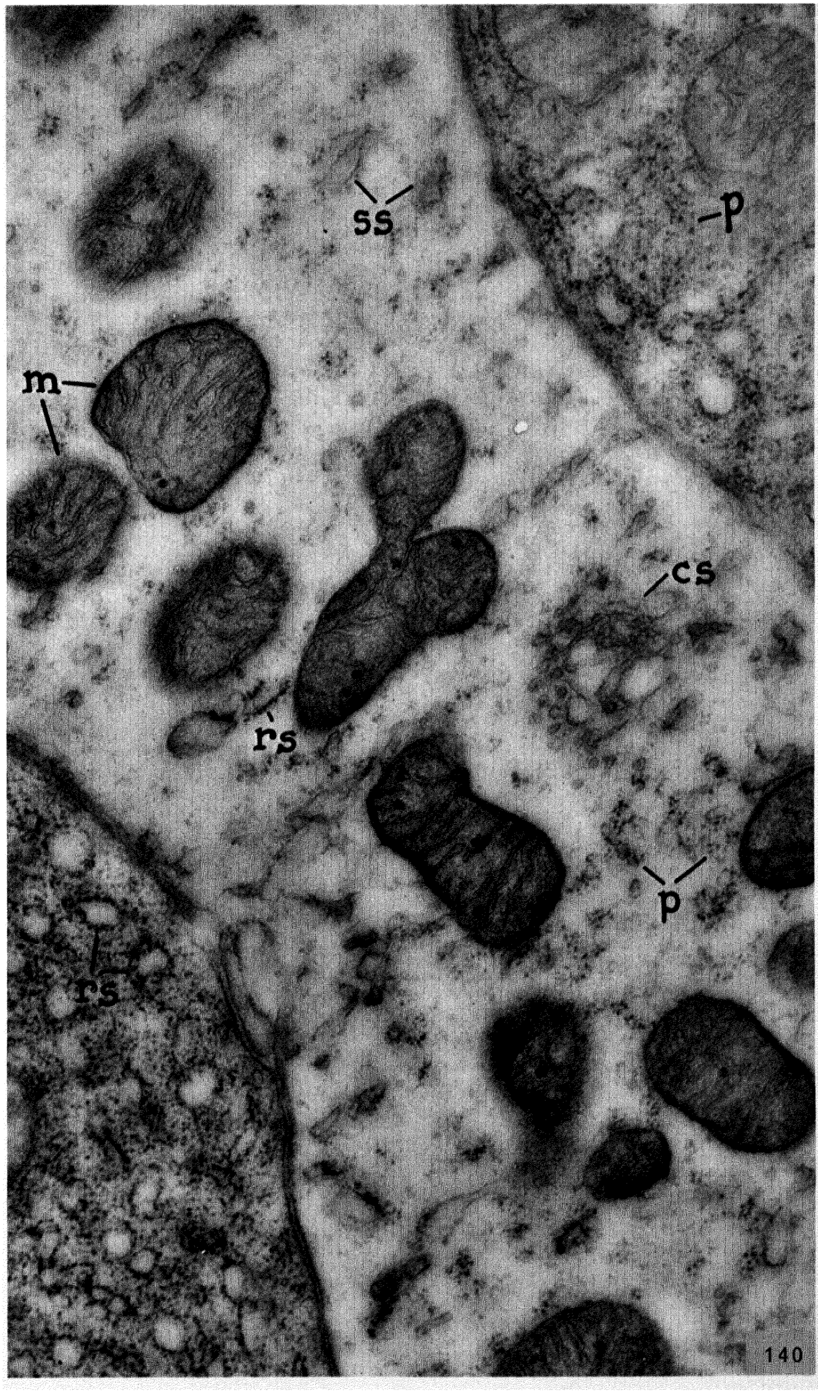

f
cp
v
cf
p
v
bm
c
m
f
p
n
bm
139

140. The micrograph shows in close juxtaposition in a pancreatic acinus parts of the cytoplasm of three cells known to have different staining affinities.

The cell in the diagonal middle field is a centro-acinar cell characterized by acidophilic cytoplasm. Its cytoplasm contains relatively few elements of the endoplasmic reticulum belonging to both the rough-surfaced (*rs*) and smooth-surfaced (*ss*) variety, the latter being more numerous. The number of particles freely scattered (*p*) in the cytoplasm is small.

By contrast, two adjacent acinar cells which appear in the upper right and lower left corners contain particles in large numbers both attached to the membrane of ER elements (*rs*) and freely scattered (*p*) in the cytoplasmic matrix. The acinar cells are known for the intense basophilia of their basal cytoplasm (ergastoplasm).

Note the numerous large mitochondria (*m*) in the centro-acinar cell and the pile of cisternae and small vesicles of its centrosphere region (*cs*). × 38,000.


ss
p
m
cs
rs
p
rs
140

141. Relatively large field in the cytoplasm of a fibroblast (rabbit myocardium).

The cell membrane, obliquely sectioned, appears at *cm,* two mitochondrial profiles at *m,* and an irregular lipide inclusion at *l.*

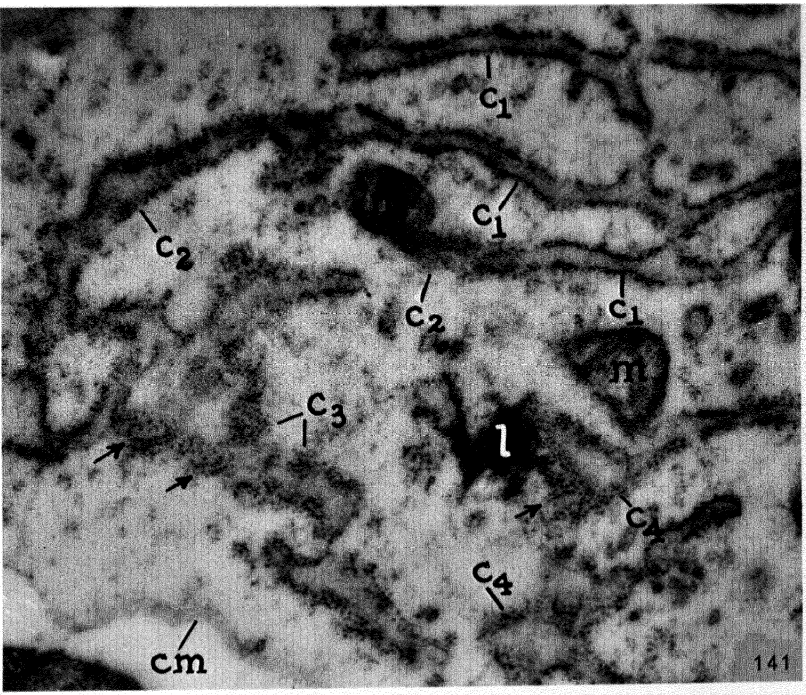
A number of elongated and irregular profiles belonging to rough-surfaced cisternae of the endoplasmic reticulum can be seen in the cytoplasm. At c_1 they are normally sectioned and consequently show their fine limiting membrane and their single layer of attached particles. At c_2 the same elements appear in slightly oblique section and finally at c_3 and c_4 relatively large areas of the cisternal membrane are seen in full-faced view because of a further increase in the obliquity of the section. Arrows indicate pairs of parallel rows of particles, a pattern frequently encountered in fibroblasts.

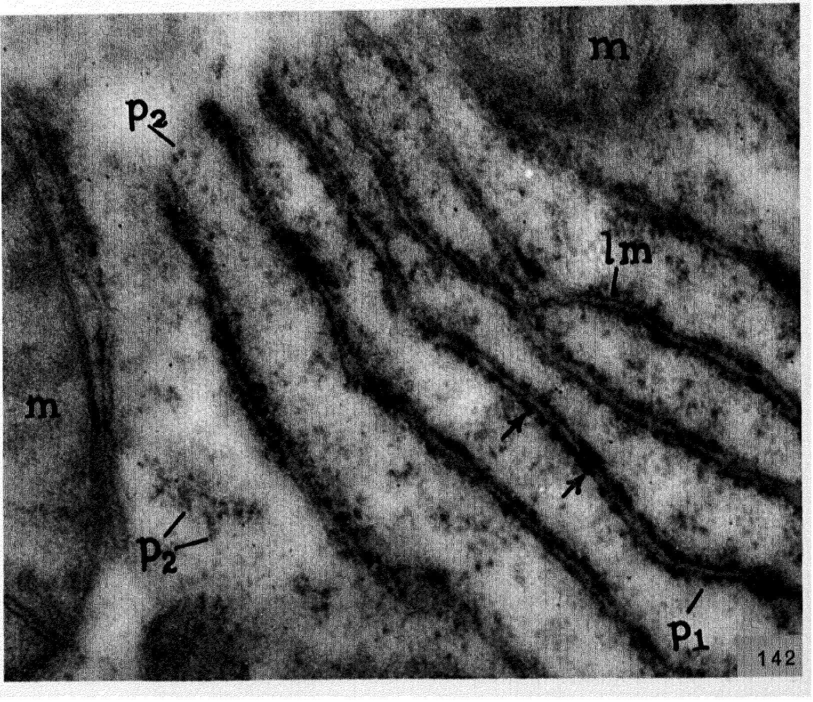
Note that the cisternae are more or less distended and that their content is of higher density than the cytoplasmic matrix. × 36,000.

142. A few profiles of normally sectioned cisternae in the cytoplasm of a parenchymal liver cell (rat).

Because of the favorable incidence of the section, the micrograph shows clearly the limiting membrane (*lm*) of the cisternae and the attached particles (p_1) which dot the membrane surface that faces the cytoplasmic matrix. Between the two arrows the attached particles are disposed in a row at more or less regular intervals (~200 A).

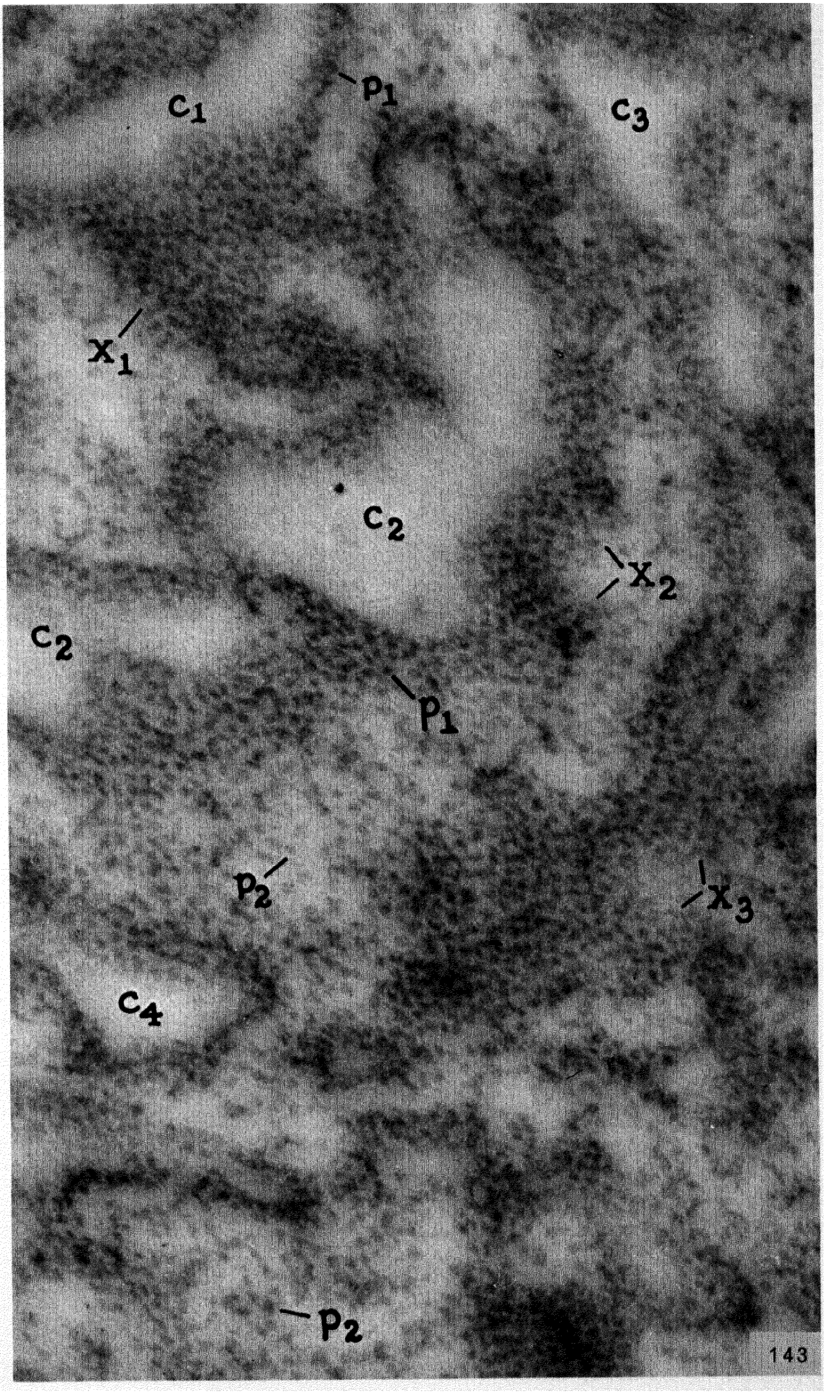
A few small dense particles (p_2) occur freely scattered in the cytoplasmic matrix. Parts of mitochondrial profiles are marked *m.* × 67,000.


c1
c1
c1
c2
c2
c3
m
l
c4
c4
cm
141


m
p2
lm
m
p2
p1
142

143. Small field in the cytoplasm of an acinar cell of the pancreas (rat). The section cuts very obliquely through a number of cisternae, whose cavities are marked c_1, c_2, c_3, and c_4, and thus makes possible a full-faced view of relatively large areas (x_1, x_2, x_3) of the cisternal membranes. Such sections give valuable information concerning the number of attached particles and their arrangement on the surface of the ER membrane. In this case it is evident that the attached particles (p_1) are exceedingly numerous and appear tightly but randomly packed.

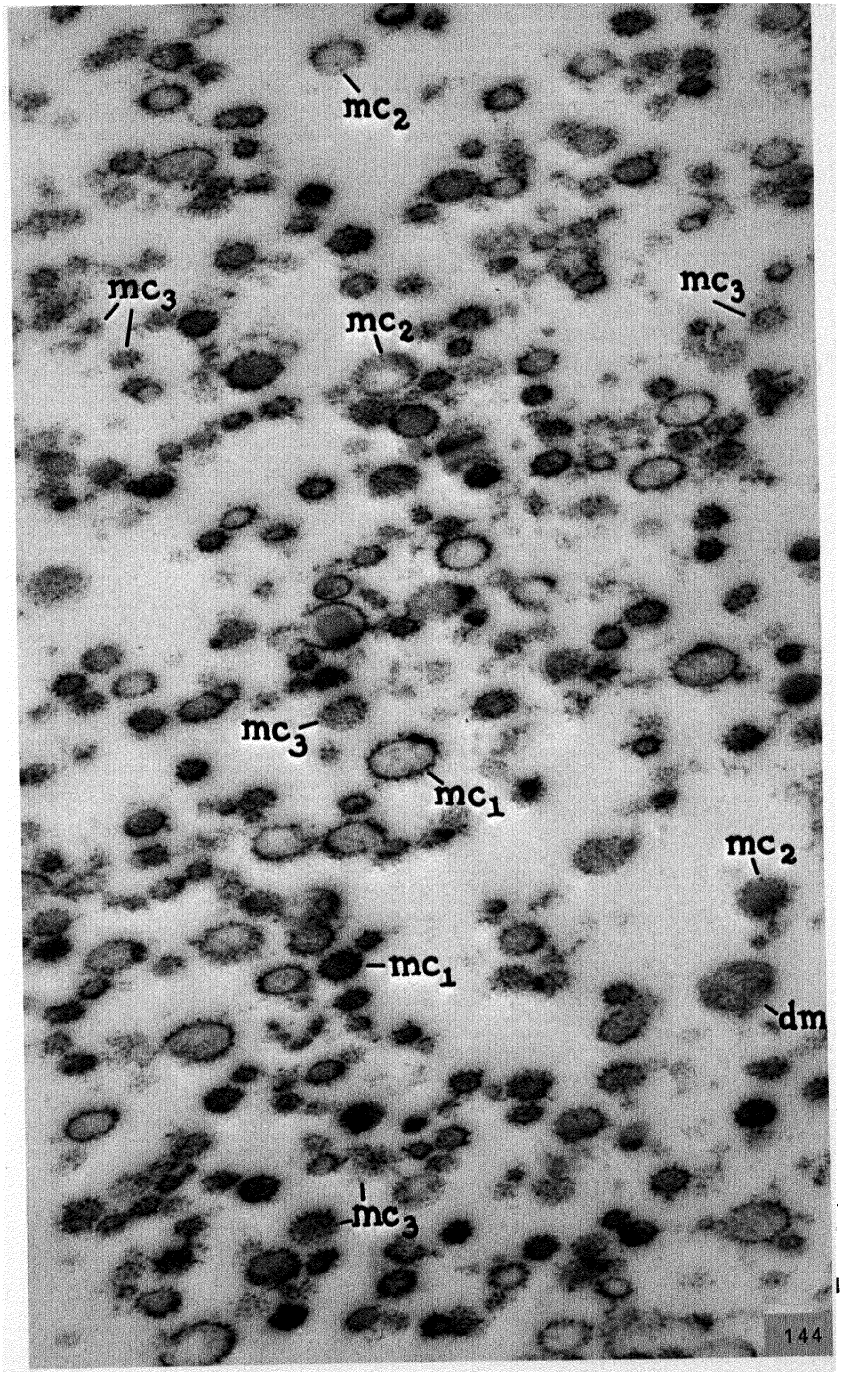
By comparison with the attached particles, freely scattered particles (p_2) in the cytoplasmic matrix are much less numerous. × 86,000.


p1
c1
c3
x1
c2
x2
c2
p1
p2
x3
c4
p2

144. Representative field in a sectioned pellet of pancreas microsomes. The microsomes were isolated (60 min. × 105,000 g) from guinea pig pancreas homogenized in 0.88 M sucrose and were fixed *in toto* in 2 per cent OsO_4 in the same concentration of sucrose. The microsomes (mc_1) consist of spherical and oval vesicles limited by a thin membrane which bears attached particles on its outer surface. These attached particles "label" the microsomes and indicate clearly that they derive from the rough-surfaced parts of the endoplasmic reticulum (cf. Fig. 135, which shows the endoplasmic reticulum *in situ* at the same magnification).

Note that many microsomes have a dense more or less homogeneous content (mc_1 and mc_2 in the lower half of the figure).

The variation encountered among the elements of the pellet is largely due to sectioning: mc_1 represents a medial section, mc_2 a lateral one, and mc_3 an extremely lateral section through microsomal vesicles. Damaged mitochondria (*dm*) and smooth-surfaced vesicles are minor contaminants or components of the microsomal fraction. × 36,000.



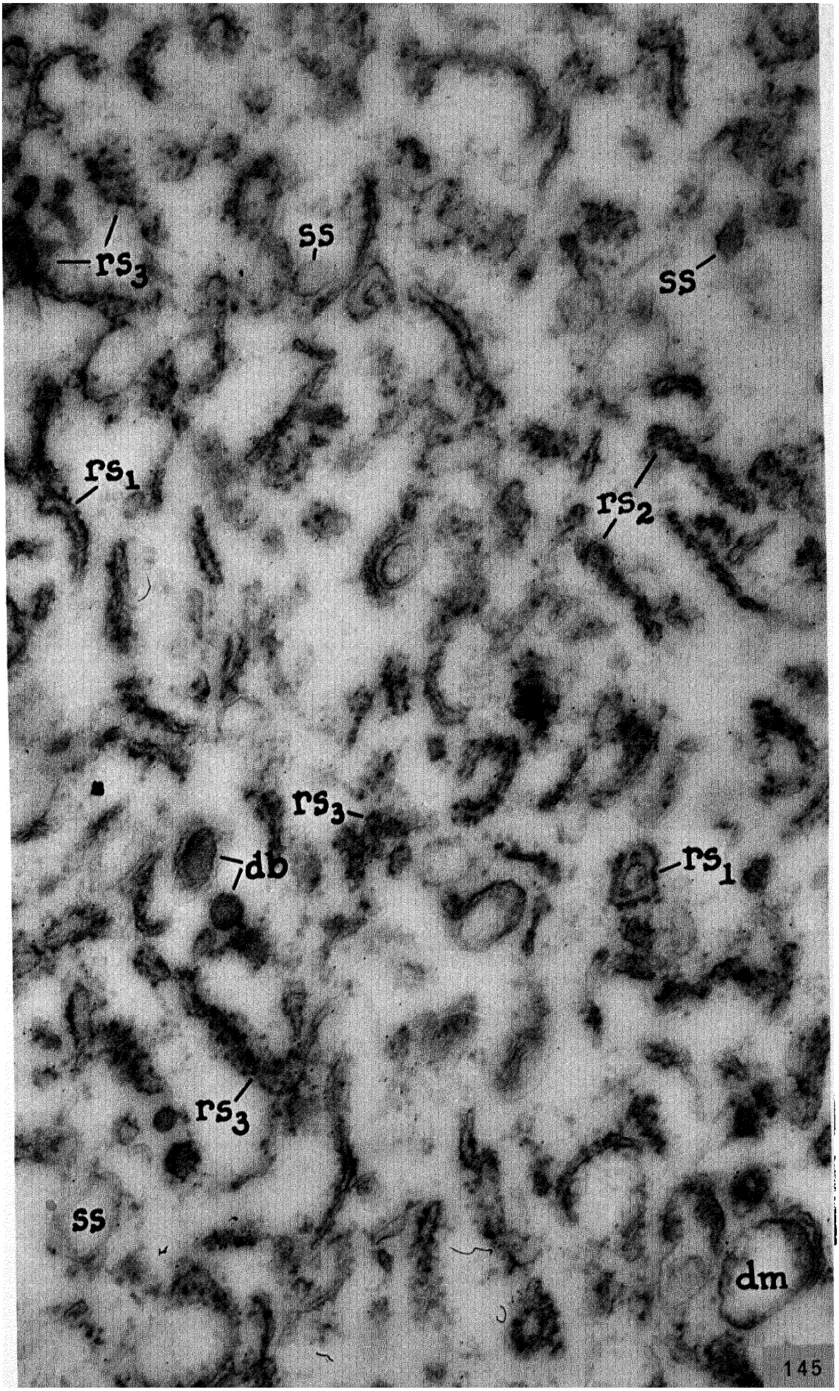
144

145. Representative field in a pellet of microsomes isolated (60 min. × 105,000 g) from a liver homogenate prepared in 0.88 M sucrose. The microsomal pellet was fixed *in toto* in 2 per cent OsO_4 in the same concentration of sucrose.

Most of the elements of this fraction appear in section as elongated profiles with particles attached on the outer surface of their membrane. These structural details indicate clearly that most microsomes derive from the rough-surfaced parts of the endoplasmic reticulum and represent fragmented cisternae. Such elements appear in normal section at rs_1 and in oblique and extremely oblique sections at rs_2 and rs_3 respectively.

Smooth-surfaced elements (*ss*), probably derived from the smooth-surfaced parts of the endoplasmic reticulum or from the centrosphere region, are also present in the microsomal pellets but their number is smaller. Dense peribiliary bodies (*db*) represent a minor component of the fraction, and damaged mitochondria (*dm*) a relatively rare contaminant.

A comparison with Figures 142 and 144 indicates clearly that: (1) liver microsomes retain the characteristic flattened appearance of intracellular cisternae (Fig. 142) when isolated and fixed in the presence of 0.88 M sucrose; and (2) under the same conditions, pancreatic cisternae fragment into small spherical vesicles which swell to a varied extent. × 55,000.

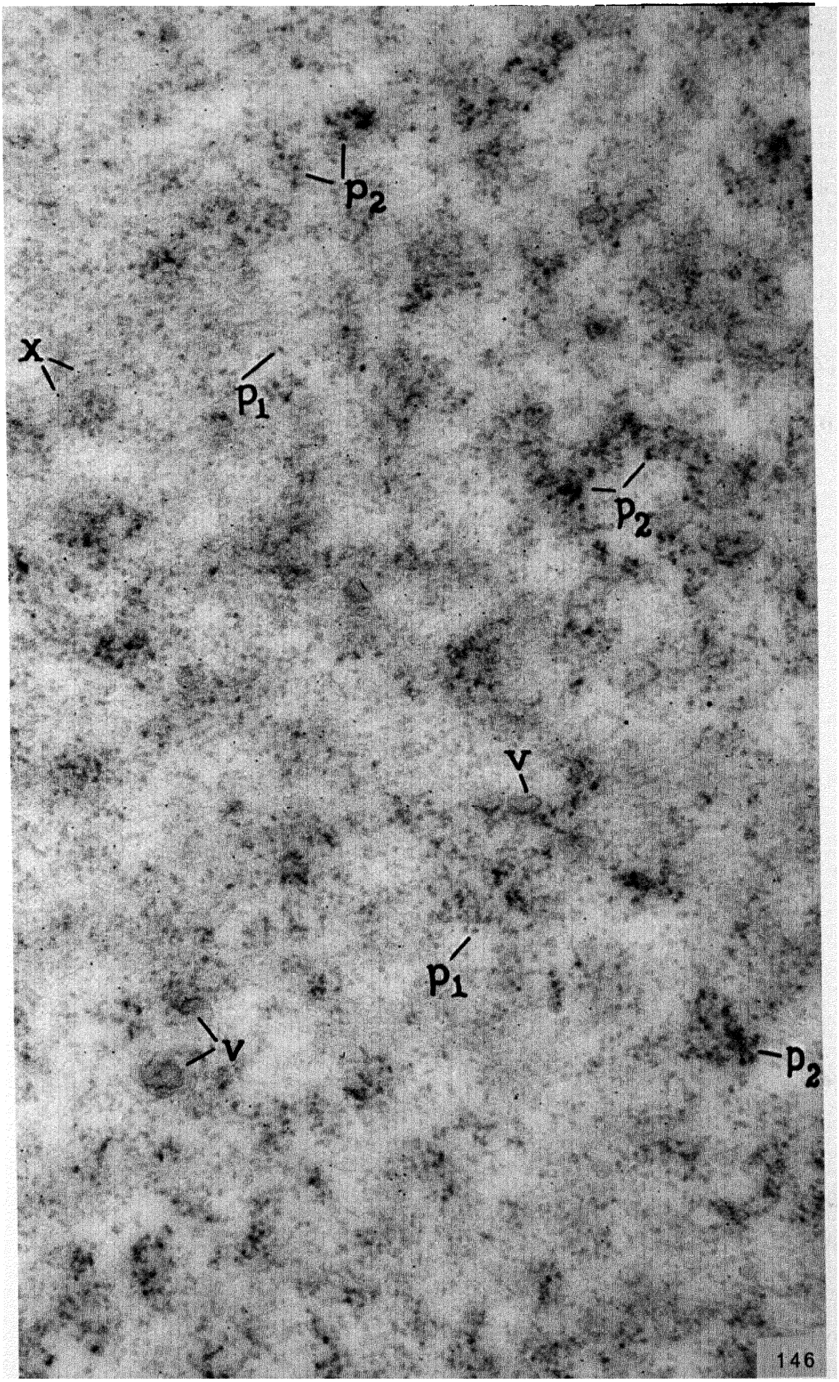

rs3
ss
ss
rs1
rs2
rs3
db
rs1
rs3
ss
dm
145

146. Pellet of hepatic microsomes treated with 0.5 per cent deoxycholate.

The microsomes were isolated (60 min. × 105,000 g) from a homogenate prepared in 0.88 M sucrose. They were treated in suspension with the concentration of deoxycholate mentioned and recentrifuged thereafter for 120 min. at 105,000 g. The pellet obtained was fixed *in toto* in 2 per cent OsO_4 in 0.15 M NaCl.

The micrograph shows that the pellet consists primarily of small dense particles which occur either isolated (p_1) or in small clusters (p_2). Only a few membrane-bound vesicles (v) are present. Their swelling during fixation in OsO_4 in an "isotonic" medium makes them more readily detectable.

A comparison with Figure 145 shows that the deoxycholate treatment damages more or less selectively the membranous component and thus permits the isolation of the particulate component of the fraction. Data in the text indicate that the particles consist primarily of ribonucleoprotein. The very dense punctate particles (x) may represent contaminating ferritin. × 55,000.

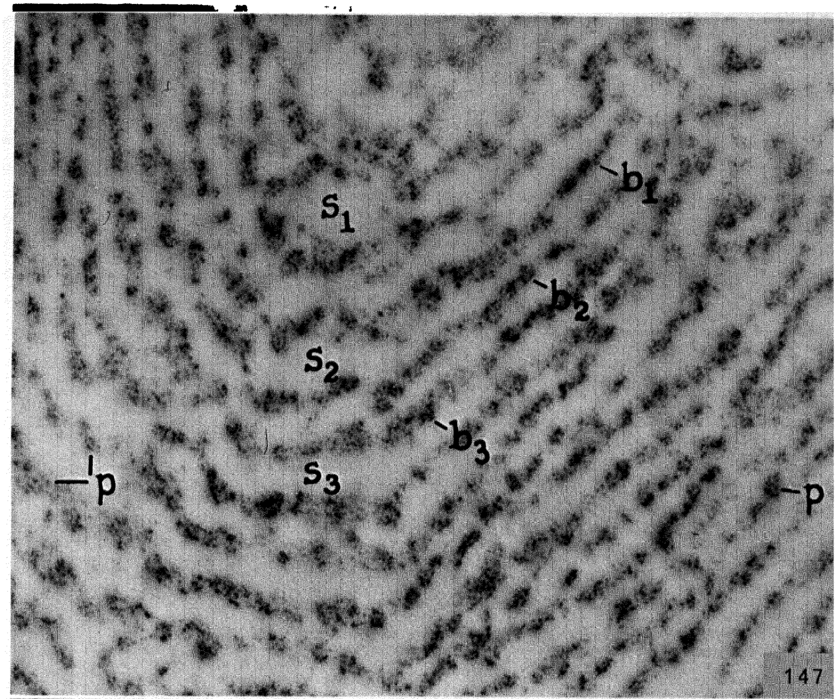

p2
x
p1
p2
v
p1
v
p2
146

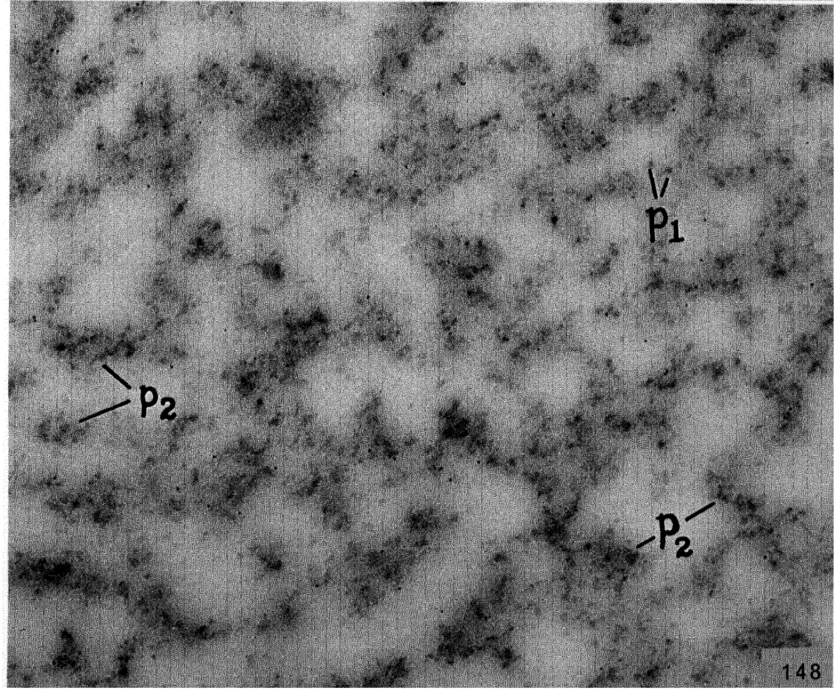
147. Relatively large field in the cytoplasm of an acinar cell of the pancreas (rat) fixed in buffered (pH 7.3) 10 per cent formalin.

The more or less parallel open spaces (s_1, s_2, s_3) represent the distended cavities of a series of cisternae of the endoplasmic reticulum. Their membrane has not been preserved by the formaldehyde fixation. The dense intervening bands (b_1, b_2, b_3) represents layers of compressed cytoplasmic matrix. Numerous small dense particles (p) can be seen within these bands. Such preparations demonstrate that the characteristic high density of the small particulate component is not due to osmium "staining." × 34,000.

148. Pellet of deoxycholate treated microsomes prepared as the specimen in Figure 146 but fixed in buffered formaldehyde.

Small dense particles isolated (p_1) or in clusters (p_2) can be recognized in the pellet. It can again be concluded that their high density is not due to $0s0_4$ "staining."


s1
b1
b2
s2
b3
s3
p
p
147

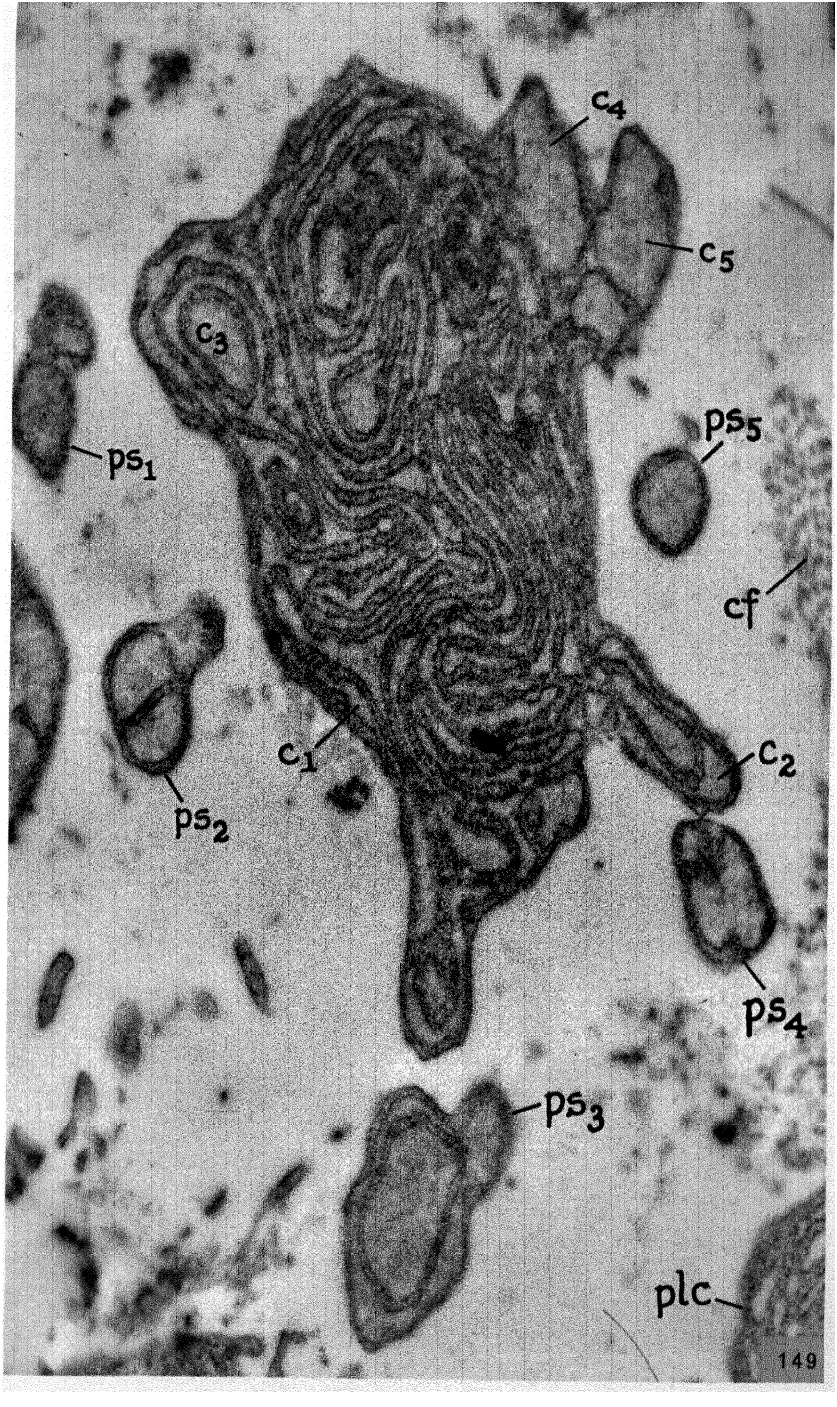

p1
p2
p2
148

149. Plasma cell in the corium of the olfactory mucosa of the rat. The section cuts laterally through the cell thus missing its nucleus and centrosphere region.

The cytoplasm is occupied by many cisternae (c_1–c_5) of the endoplasmic reticulum which show various degrees of distension and are filled with a relatively dense, and apparently amorphous material. Extremely distended cisternae marked c_4, c_5 can be seen at the periphery of the cell. The profiles marked ps_1–ps_5 represent either pseudopodia of the plasma cell in the middle of the field or sloughed off fragments of cytoplasm. All of them are filled by one or two distended cisternae of the endoplasmic reticulum.

Such appearances suggest that the cavity of the ER can be used as storage space for a cell product (globulins?).

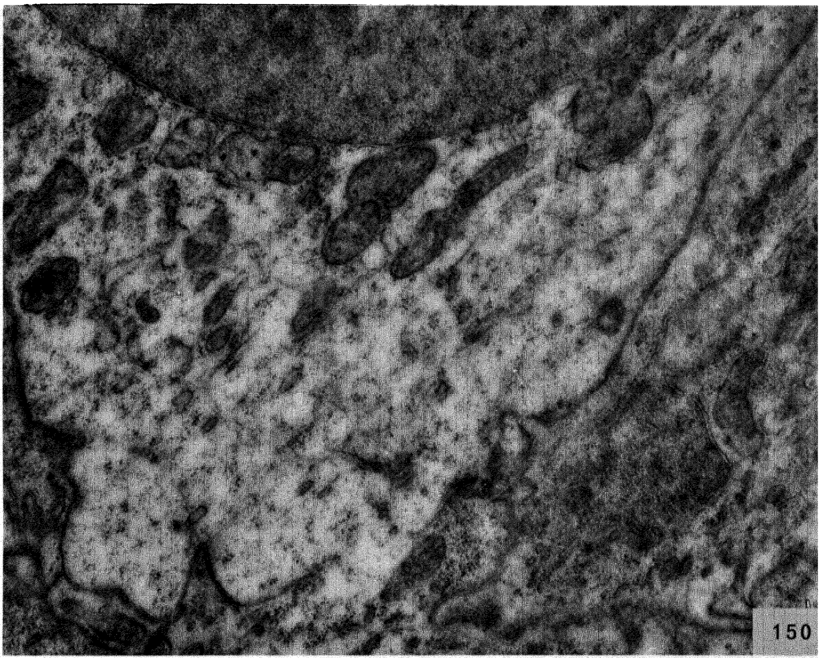
Another plasma cell appears at *plc* and a bundle of collagen fibrils at *cf*. × 25,000.


c_4
c_5
c_3
ps_5
ps_1
cf
c_1
c_2
ps_2
ps_4
ps_3
plc
149

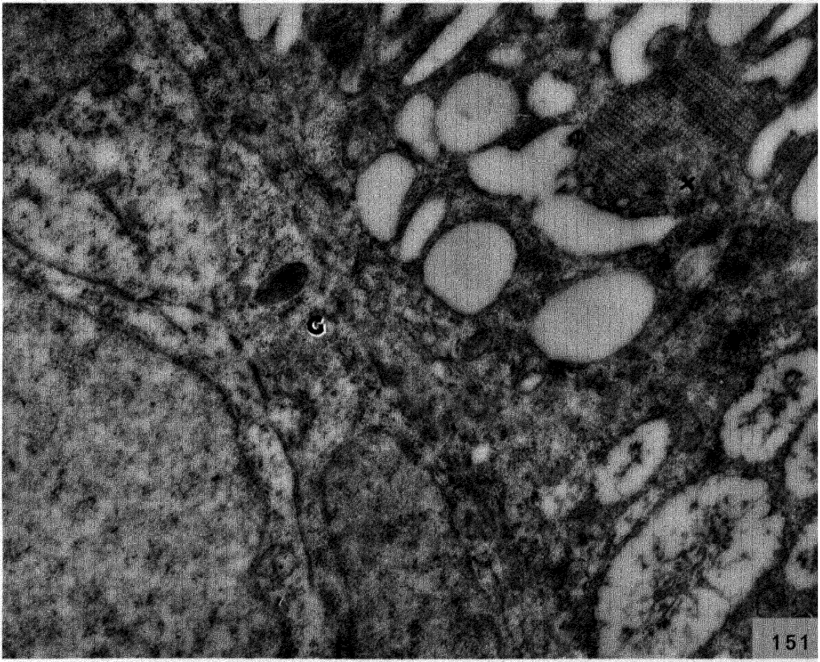
All figures are electron micrographs of thin sections of tissues fixed in 2 per cent osmium tetroxide buffered to pH 7.4 with acetate-veronal buffer and embedded in n-butyl methacrylate.

150. Portion of an undifferentiated cell in an acinus of the Meibomian gland of a rat. The nucleus, bounded by the usual double membrane, is visible at the upper margin of the picture. The cytoplasm contains numerous mitochondria and a diffusely dispersed ergastoplasm consisting of fine, dense granules and a few rounded and elongated profiles of the endoplasmic reticulum. Thin fibrils extend in all directions through the cytoplasm. The right quarter of the picture contains part of an obliquely sectioned cell with a densely granular nucleus. × 23,500.

151. Portions of several adjacent cells in an acinus of the Meibomian gland of a rat. The three cells to the left of the figure are relatively undifferentiated and the two cells to the right are more differentiated. Near the center of the picture is a collection of agranular profiles (G) that represent the small Golgi complex of the undifferentiated cells. In the lower right corner, the cytoplasm contains several secretory spaces with clumped strands or tubules of agranular reticulum. The upper right corner contains many circular profiles of secretory spaces from which the oily secretory product has been dissolved out. Each space is bounded by a wall of agranular reticulum, which is seen in tangential section at x. This wall is presented at high magnification in Figure 154. × 19,200.



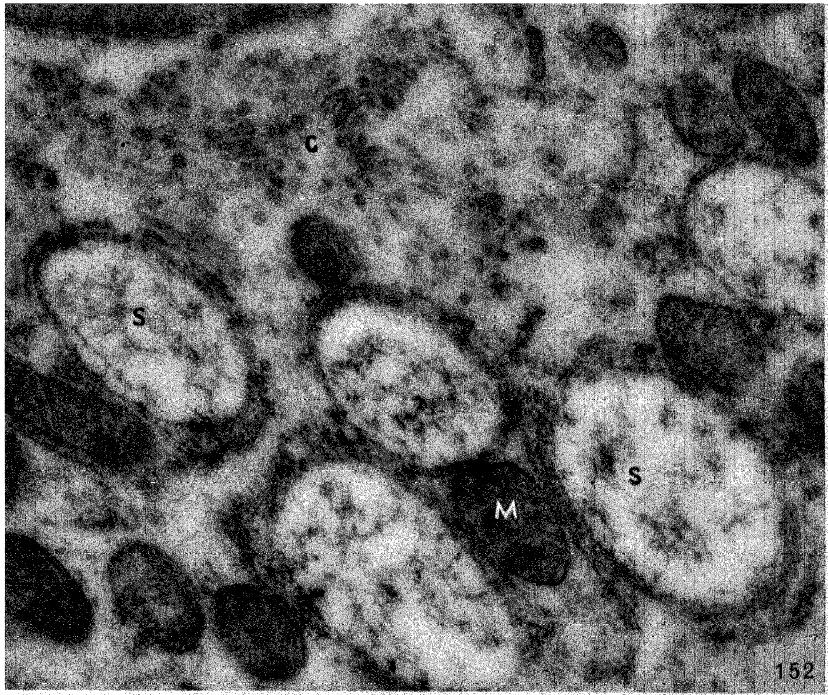
150



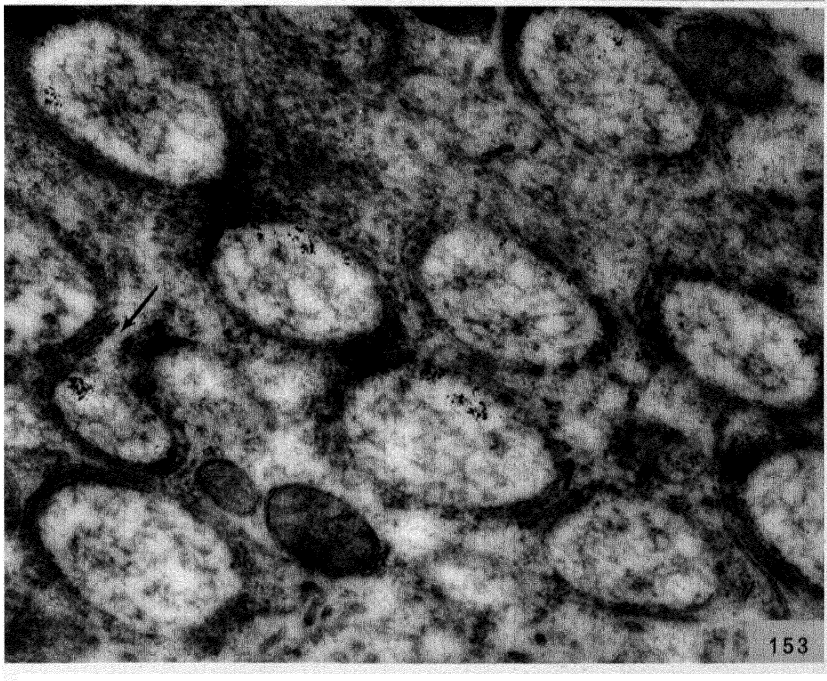
151

152. Portion of an intermediate cell in an acinus of the Meibomian gland of a rat. The edge of the nucleus lies at the upper left corner of the figure and below it are the clustered tubular profiles of the Golgi complex (G). The remainder of the picture contains five secretory droplets (S) and numerous mitochondria (M). Each droplet is surrounded by a husk of agranular reticulum in the form of circularly arranged tubules or cisternae. The secretory product has been dissolved out. × 44,500.

153. Portion of the cytoplasm in an intermediate cell from an acinus of the Meibomian gland of a rat. Numerous secretory spaces, each surrounded by a spherical husk of agranular reticulum or Golgi membranes, fill the cytoplasm. In the interstices between droplets are hordes of tubular profiles also belonging to the Golgi complex. The formation of the secretory spaces by folding of the agranular reticulum into a sphere is indicated by the arrow. × 35,700.

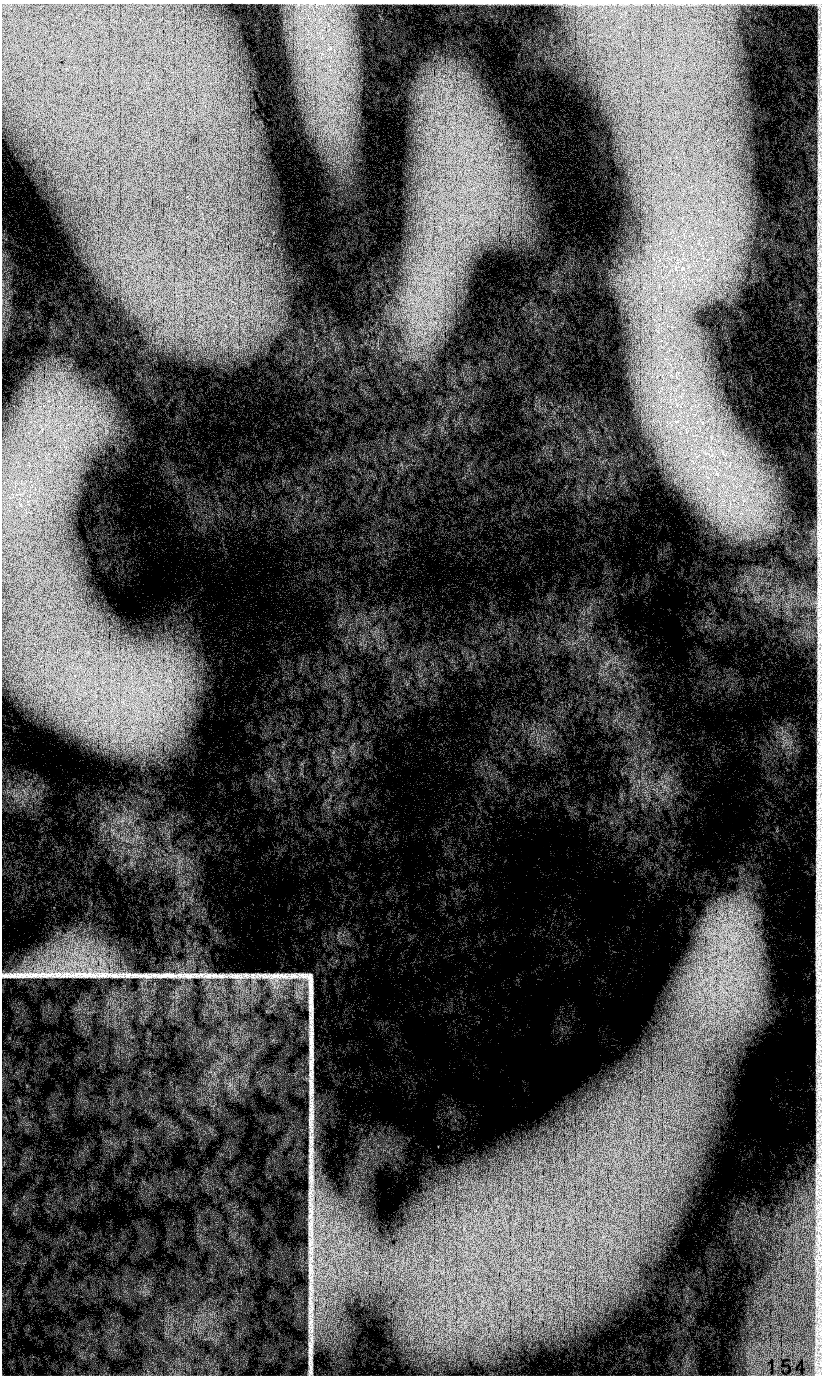


152

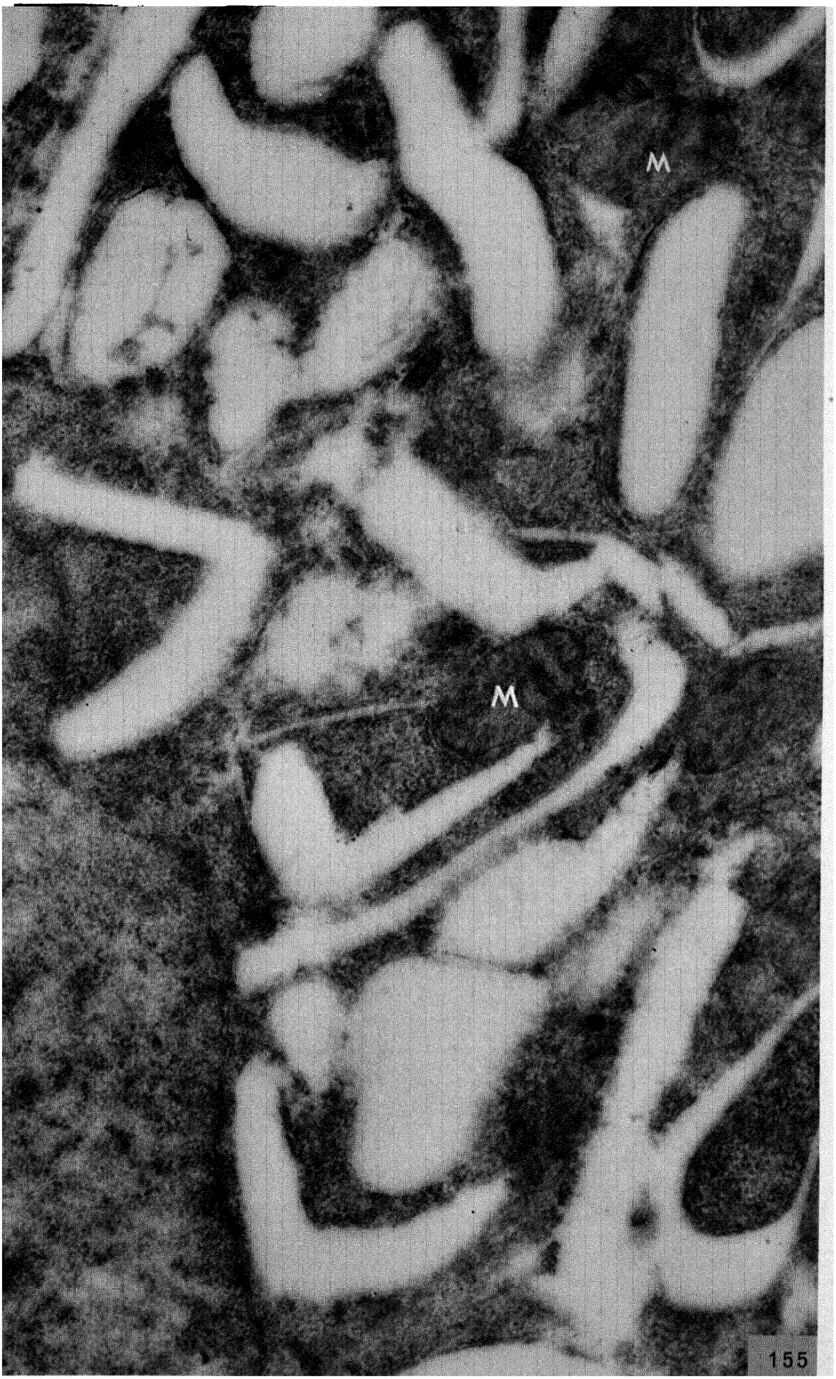


153

154. Tangential section of the Golgi membranes forming the husk about a secretory space in the cytoplasm of a cell from the Meibomian gland of a rat (see Fig. 151). The wall of the secretory space consists of many, closely apposed, undulating, fenestrated, anastomosing, membrane-delimited cisternae. The inset shows the lower left portion of this wall at a higher magnification. Here the continuity of the cisternae can be followed for short distances and the anastomoses between them may be seen. × 84,100; inset, × 157,900.

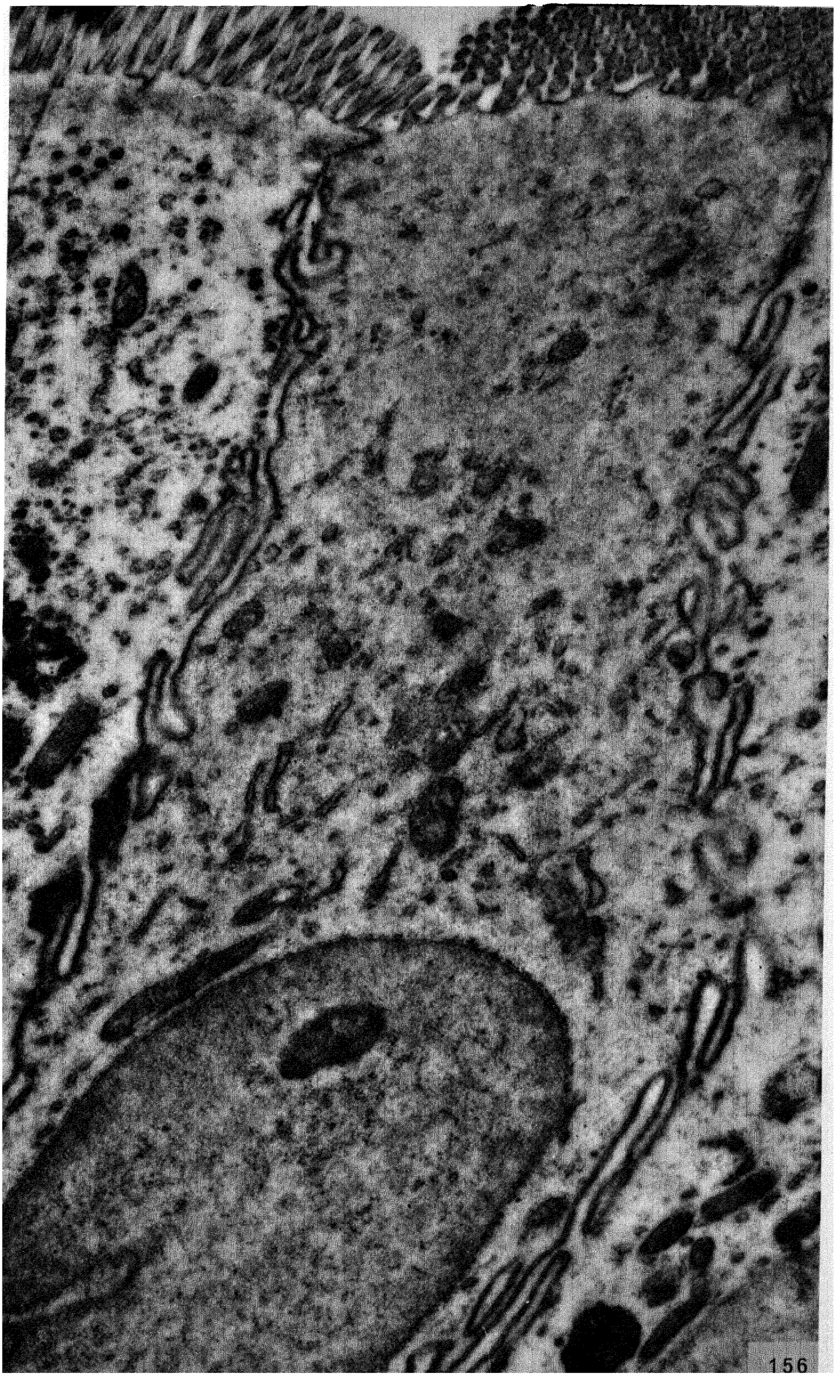

154

155. Portion of mature cell in an acinus of the Meibomian gland of a rat. Part of the nucleus appears at the lower left of the figure. The cytoplasm is extremely dense and granular. It is permeated by irregular clefts and oval spaces from which the secretory product has been dissolved out. Healthy mitochondria (M) are visible here and there between the secretory droplets. × 42,000.



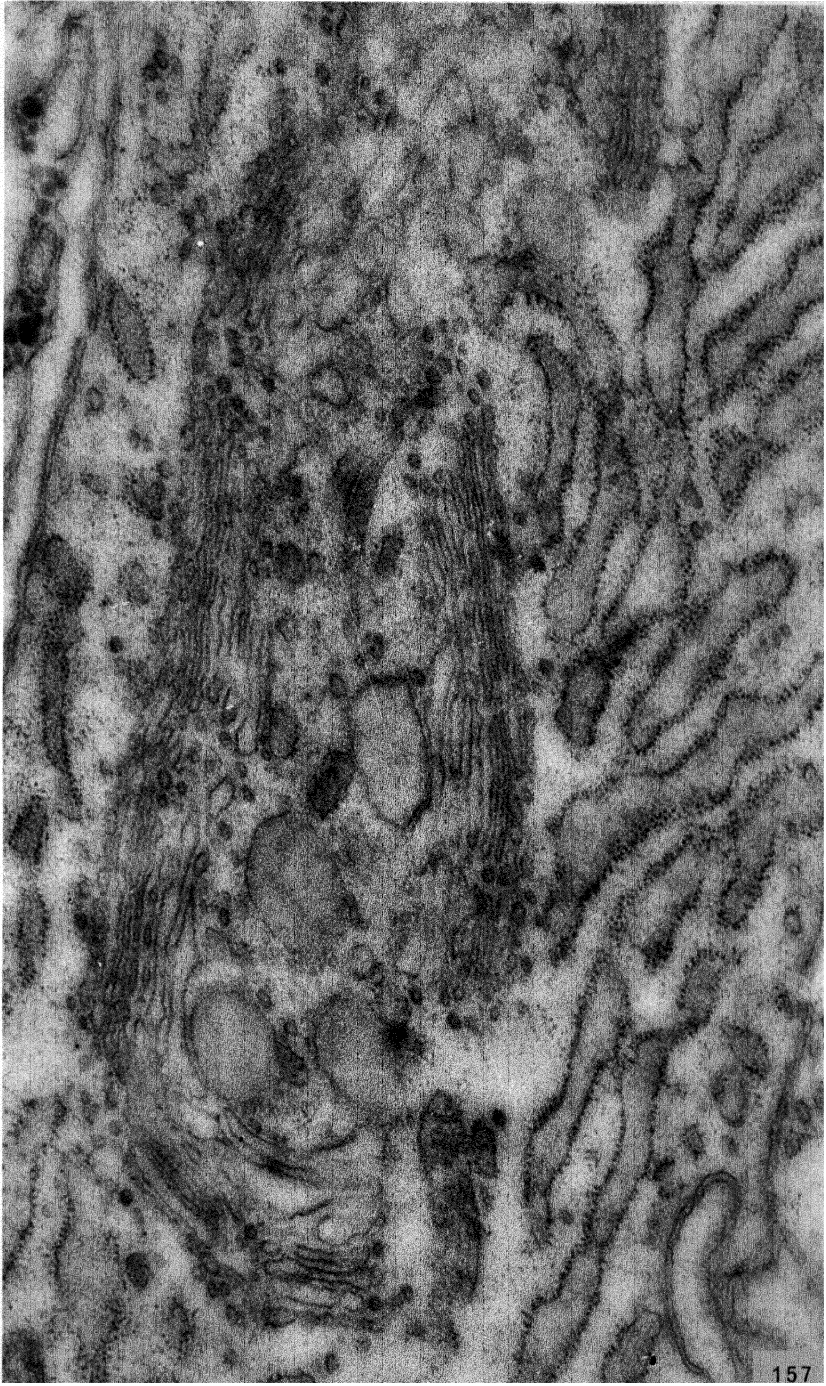
155

156. Resting goblet cell in the epithelium covering a villus in the jejunum of a rat. The indented oval nucleus lying at the base of the cell contains a prominent, dense nucleolus. The supranuclear cytoplasm contains mitochondria, ergastoplasm, and occasional agranular elements of the Golgi complex. The apical cytoplasm is full of densely matted, fine fibrils, which give the cell a dark appearance contrasting with that of the neighboring absorptive cells. The apical surface of the cell is surmounted by a striate border consisting of microvilli exactly like that of its neighbors. Notice the multiple, interdigitating plications of the lateral surfaces of adjacent cells. The extremely dense, round masses in the absorptive cells on either side of the goblet cell are absorbed fat droplets. × 19,000.



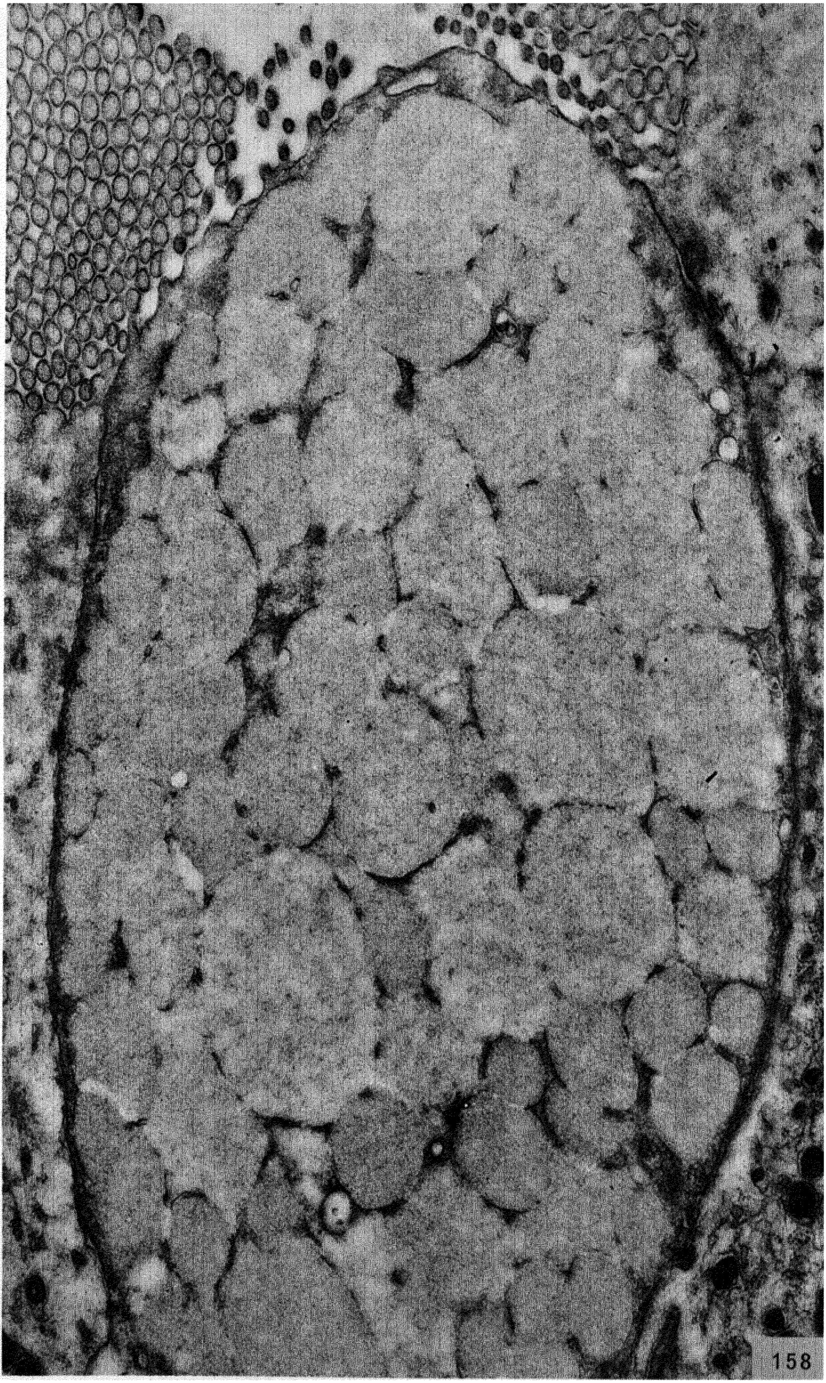
156

157. Golgi complex in the supranuclear cytoplasm of a goblet cell from the jejunal epithelium of a rat. The nucleus lies below the Golgi complex and is not included in the figure. The theca of the goblet, lying above the Golgi complex, is also excluded. The Golgi complex consists of piles of closely apposed, broad, flattened, fenestrated cisternae and small vesicles arranged in a flasklike array. Small and large secretory droplets lie in the center of the array. Near the top of the figure the section passes obliquely through the cisternae and reveals a number of anastomoses or branchings. The contrast in arrangement between the granular endoplasmic reticulum of the ergastoplasm (right of figure) and the agranular reticulum of the Golgi complex is evident. × 40,600.

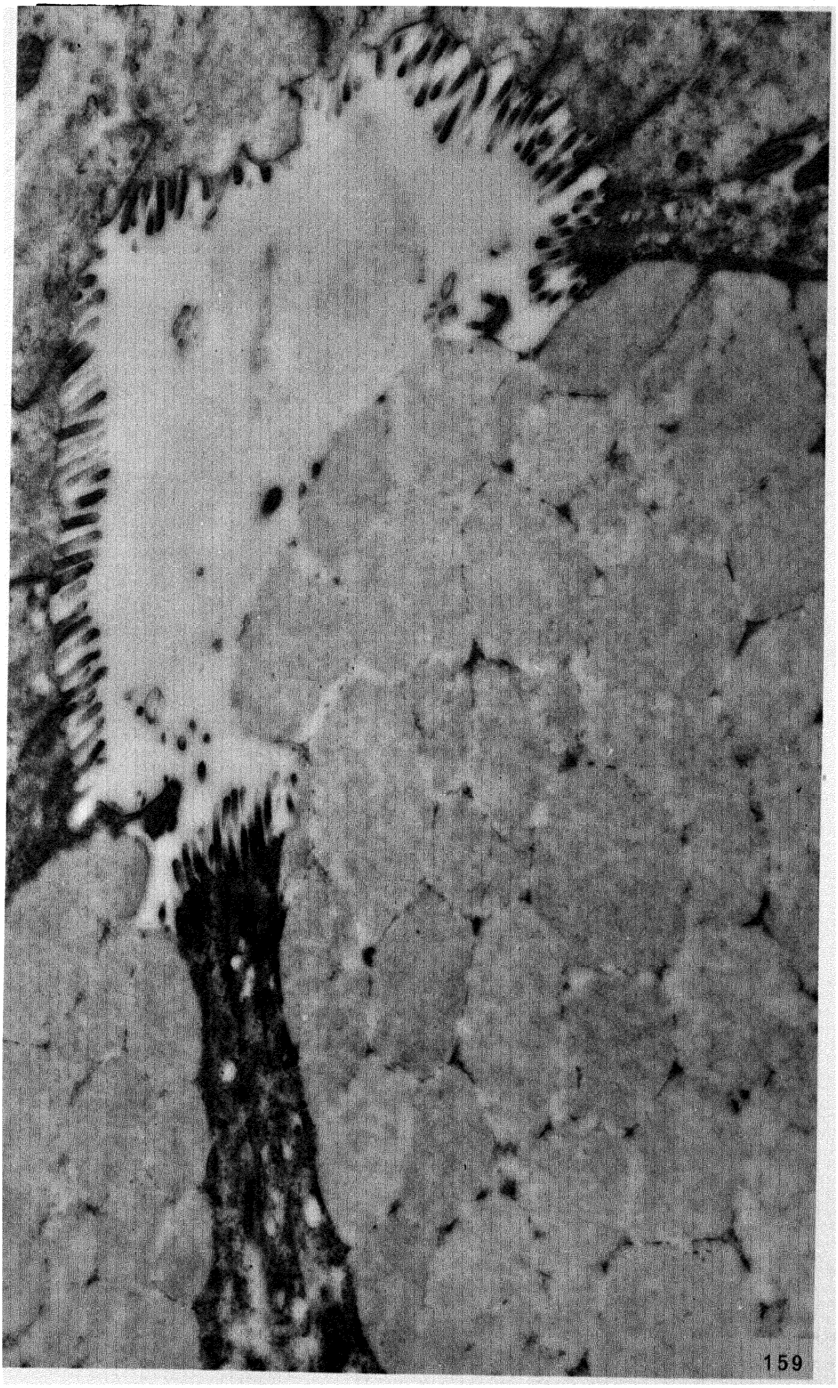


157

158. Apex of a mature goblet cell from the jejunal epithelium of a rat. The droplets of mucus distend the theca of the goblet. Most of them are discrete and are surrounded by fine membranes, but some of them have coalesced. The plications of the lateral cell surfaces have flattened out, and the microvilli of the striate border are much less prominent than in Figure 156. × 27,500.


158

159. Apex of a mature goblet cell from the mouth of a crypt in the jejunum of a rat. The droplets of mucus are still discrete but most of them are not surrounded by intact membranes. The apical plasmalemma has burst, and mucus is oozing out into the lumen of the crypt. × 19,500.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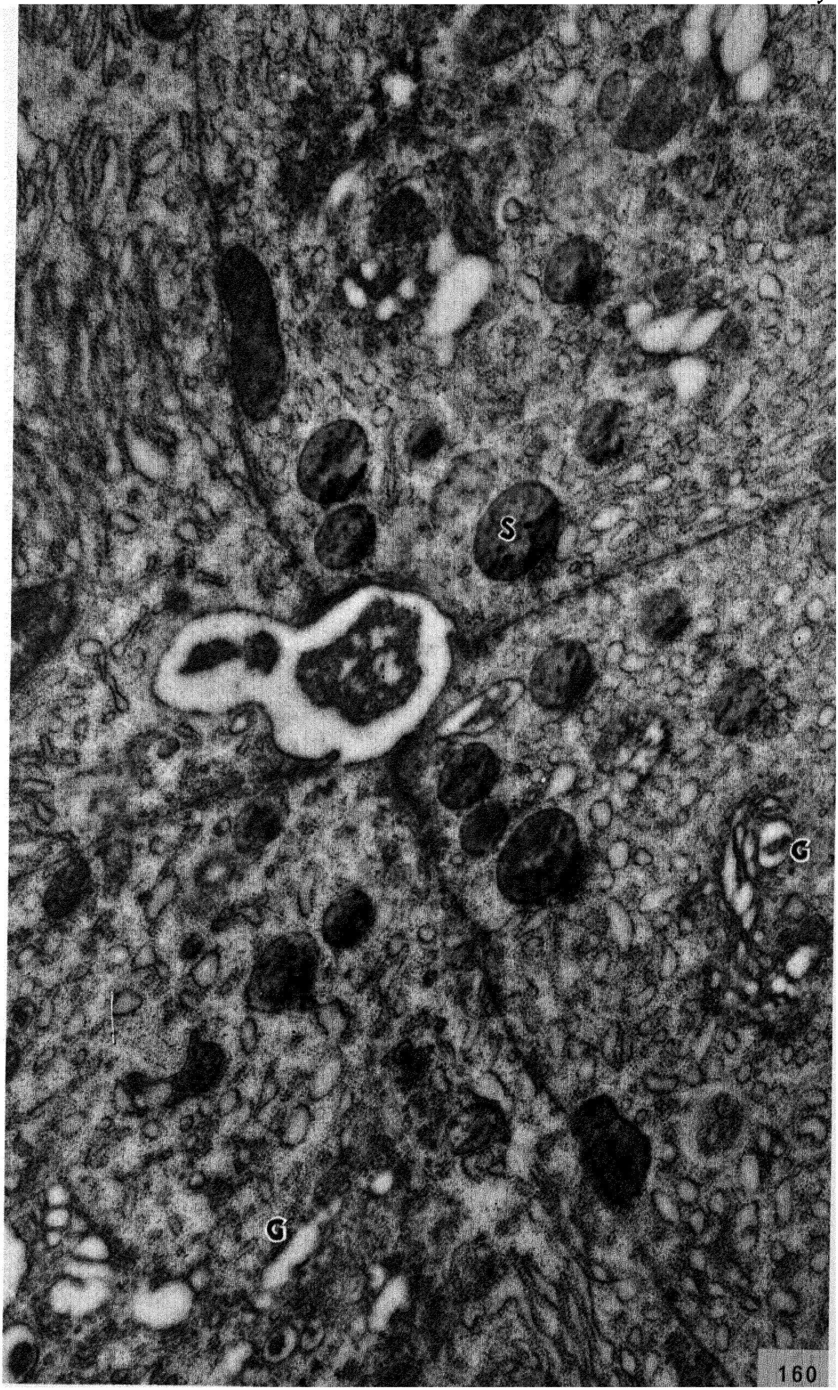
160. Apices of neighboring acinar cells in the pancreas of a mouse that had received a subcutaneous injection of 2 mg of pilocarpine 4 hours before autopsy. Most of the secretory granules (S) have disappeared but a few remain near the apices of the cells. The heterogeneous densities in these granules are artifacts of sectioning. The ergastoplasm of each cell consists of small, round, and elongated profiles of endoplasmic reticulum studded with fine nucleoprotein granules. The Golgi complex (G) of each cell consists of flat and dilated agranular cisternae and small vesicles closely packed together. Some of the dilated cisternae contain irregular dense masses which represent accumulations of secretory material. The lumen of the acinus contains some flocculated, extruded, secretory product. × 19,500.



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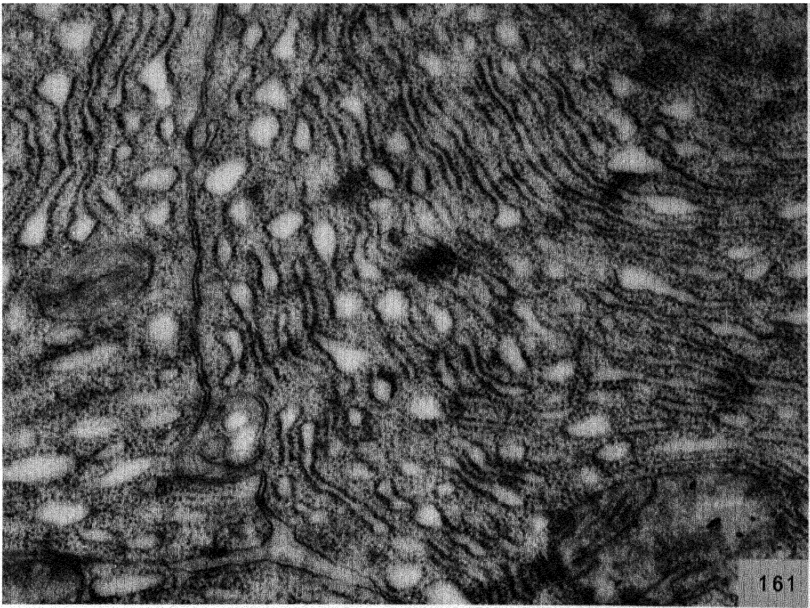
161. Ergastoplasm in the bases of two adjacent acinar cells in the pancreas of an untreated, fasted rat. The ergastoplasm consists of highly oriented membrane-delimited cisternae or sacs (the endoplasmic reticulum) and encrustations of fine, dense, nucleoprotein granules. The sacs are disposed in a parallel fashion about the nucleus, which is just visible at the upper right corner of the picture. The apposing plasma membranes of the two cells are represented by the sinuous double line which passes vertically through the left third of the figure. × 34,300.

162. Golgi complex of a pancreatic acinar cell from a mouse that had received 2 mg of pilocarpine subcutaneously 4 hours before autopsy. The closely packed agranular cisternae display frequent dilatations. Swarms of vesicles surround the cisternal packets and many vesicles contain homogeneous dense secretory material. × 34,300.



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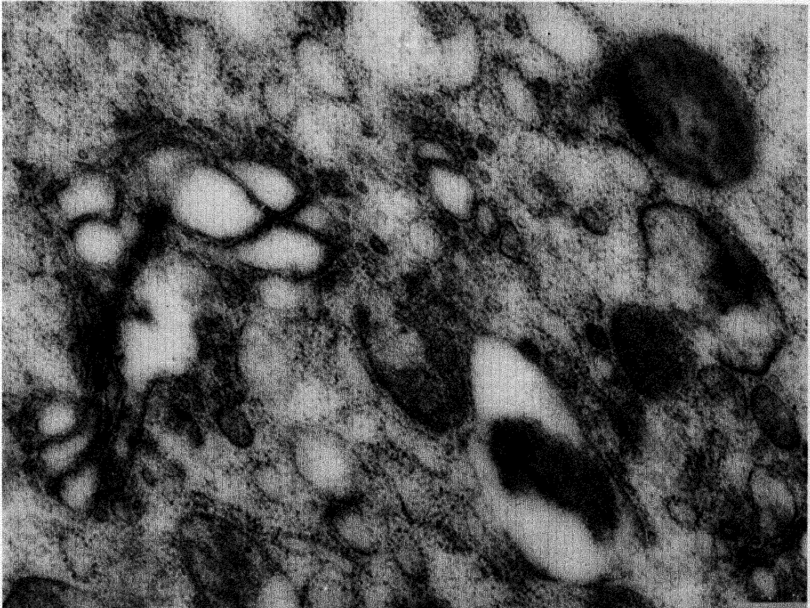

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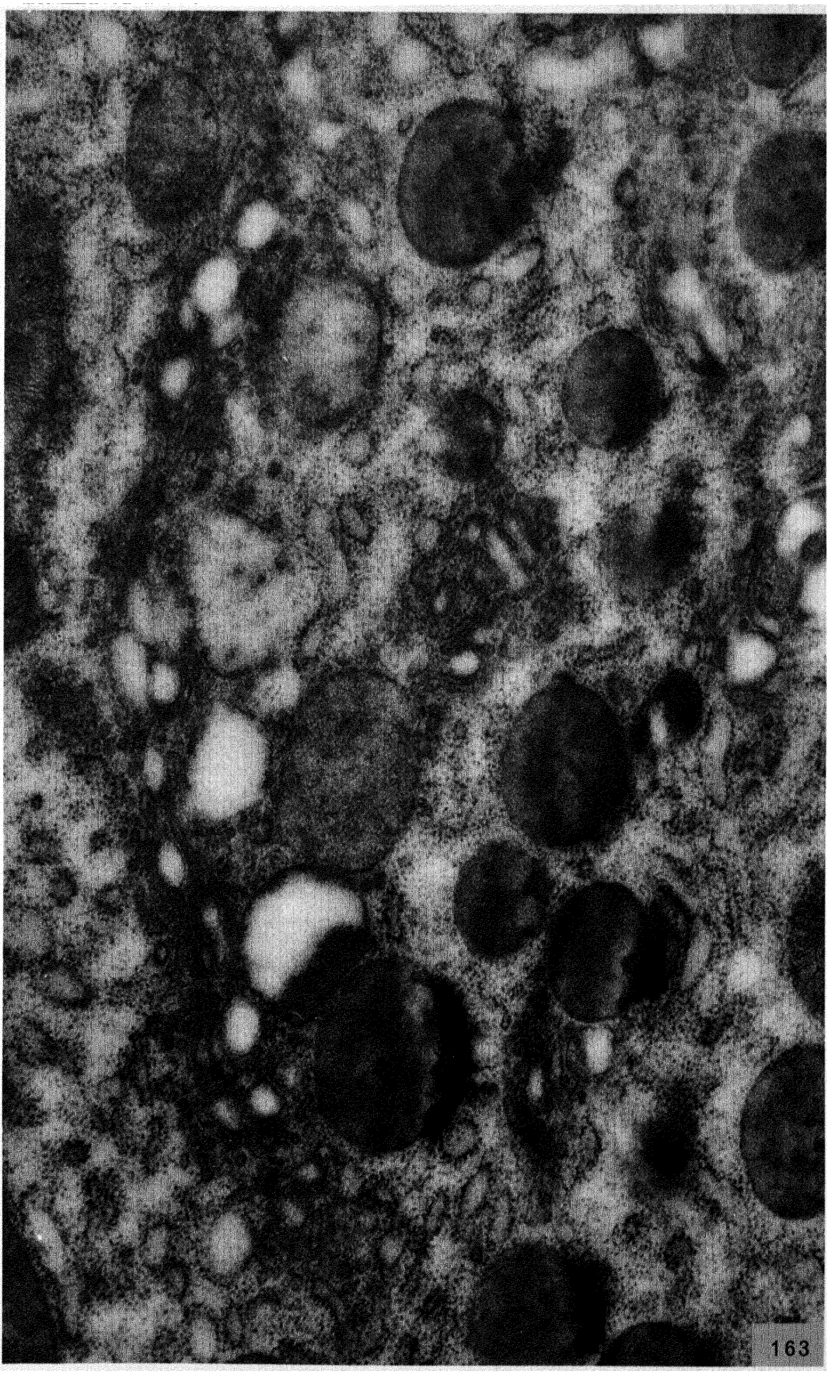
163. Golgi complex of a pancreatic acinar cell from a mouse that had received 2 mg of pilocarpine subcutaneously 4 hours before autopsy. The Golgi complex in this instance has a large proportion of vesicular profiles. Numerous mature and immature secretory droplets are visible and a series of intermediate forms can be constructed, grading from the empty small vesicles to the mature droplets. × 40,200.



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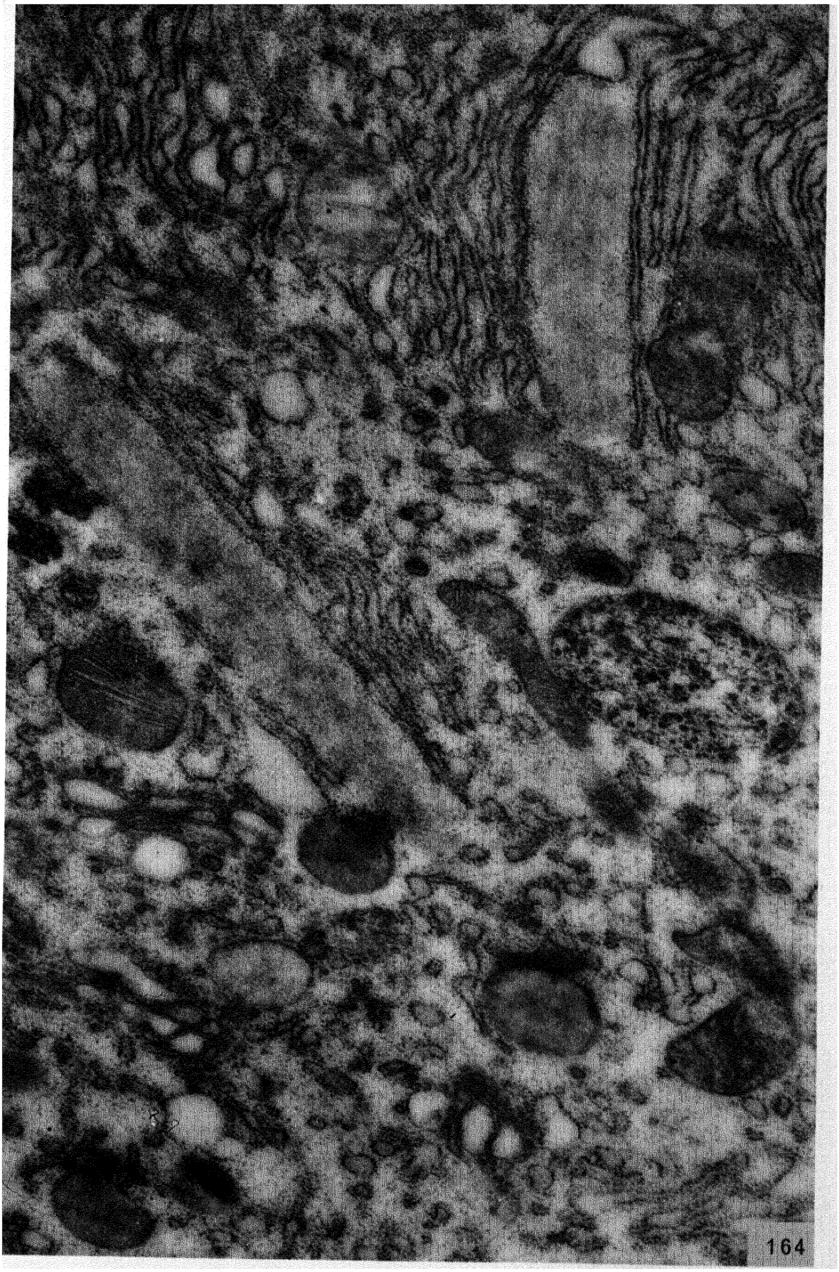
164. Paranuclear cytoplasm of a pancreatic acinar cell from a mouse that had received 2 mg of pilocarpine subcutaneously 4 hours before autopsy. Two prominent, pale, elongate masses represent regions of focal disintegration of the ergastoplasm, which are frequent in pancreatic cells during the reaction to pilocarpine treatment. The large, oval, membrane-limited aggregation of dense granules and membranes at the right margin of the figure is also a frequent formation under these conditions. The ergastoplasm lacks the schematic parallel orientation of the broad sacs which characterizes the resting cell. Parts of the Golgi complex and secretory droplets are seen at the lower left portion of the figure. × 26,000.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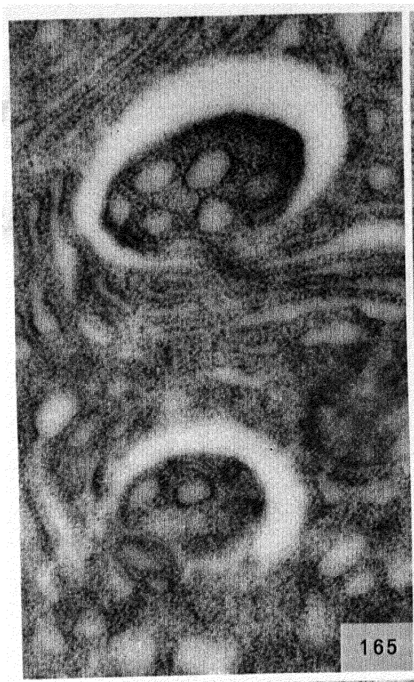
165. Ergastoplasmic formation in the cytoplasm of a pancreatic acinar cell from a mouse that had received 2 mg of pilocarpine 4 hours before autopsy. These two formations consist of ergastoplasmic vesicles and their associated nucleoprotein granules, which have condensed into round masses separated by a space from the remaining ergastoplasm. Such formations are frequent in pancreatic acinar cells of pilocarpine-treated animals. × 34,600.

166. New formations in the cytoplasm of a pancreatic acinar cell from a mouse that had received 2 mg of pilocarpine subcutaneously 4 hours before autopsy. Two of these formations consist of pale, homogeneous material containing delicate circular membranous profiles and a shell of extremely dense heavy membranes. The third formation is a dense aggregation of fine granules. Vesicles of the Golgi complex lie at the left border of the figure. × 33,500.

167. Portion of an acidophile cell in the adenohypophysis of a normal male rat. The nucleus, bounded by a double membrane, lies at the upper border of the picture. Secretory droplets are disposed about the other margins of the figure and in the central area is a well-developed Golgi complex. Piles of agranular cisternae are arranged about a roughly circular area enclosing myriad vesicles and secretory granules of various sizes. A clavate mass of heterogeneous density bounded by a single membrane is visible near the center of the figure. Around the Golgi complex are a few mitochondria and elements of the ergastoplasm. × 29,000.



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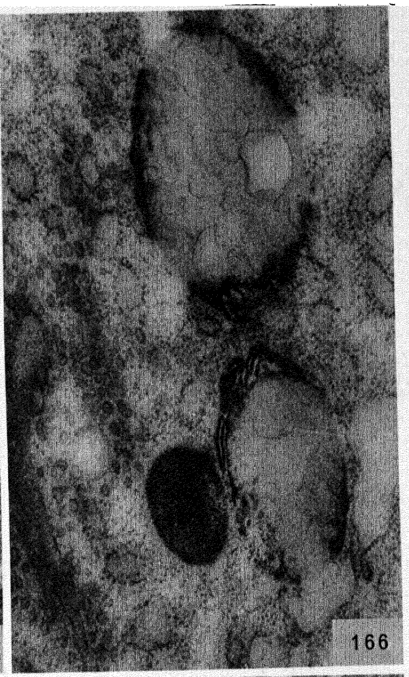

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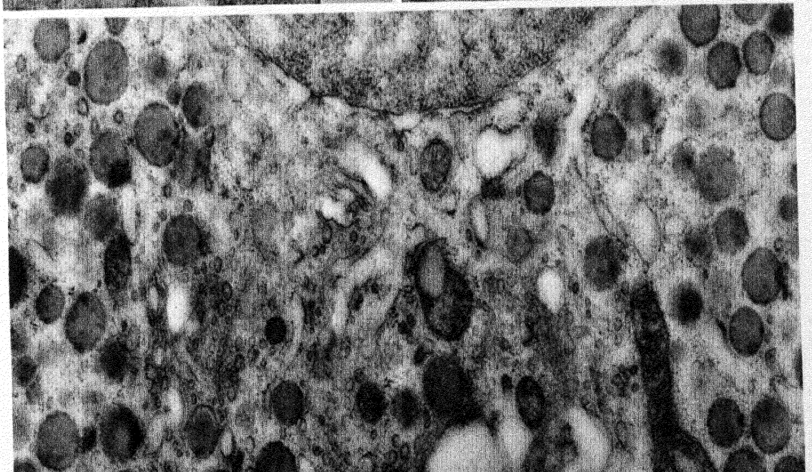

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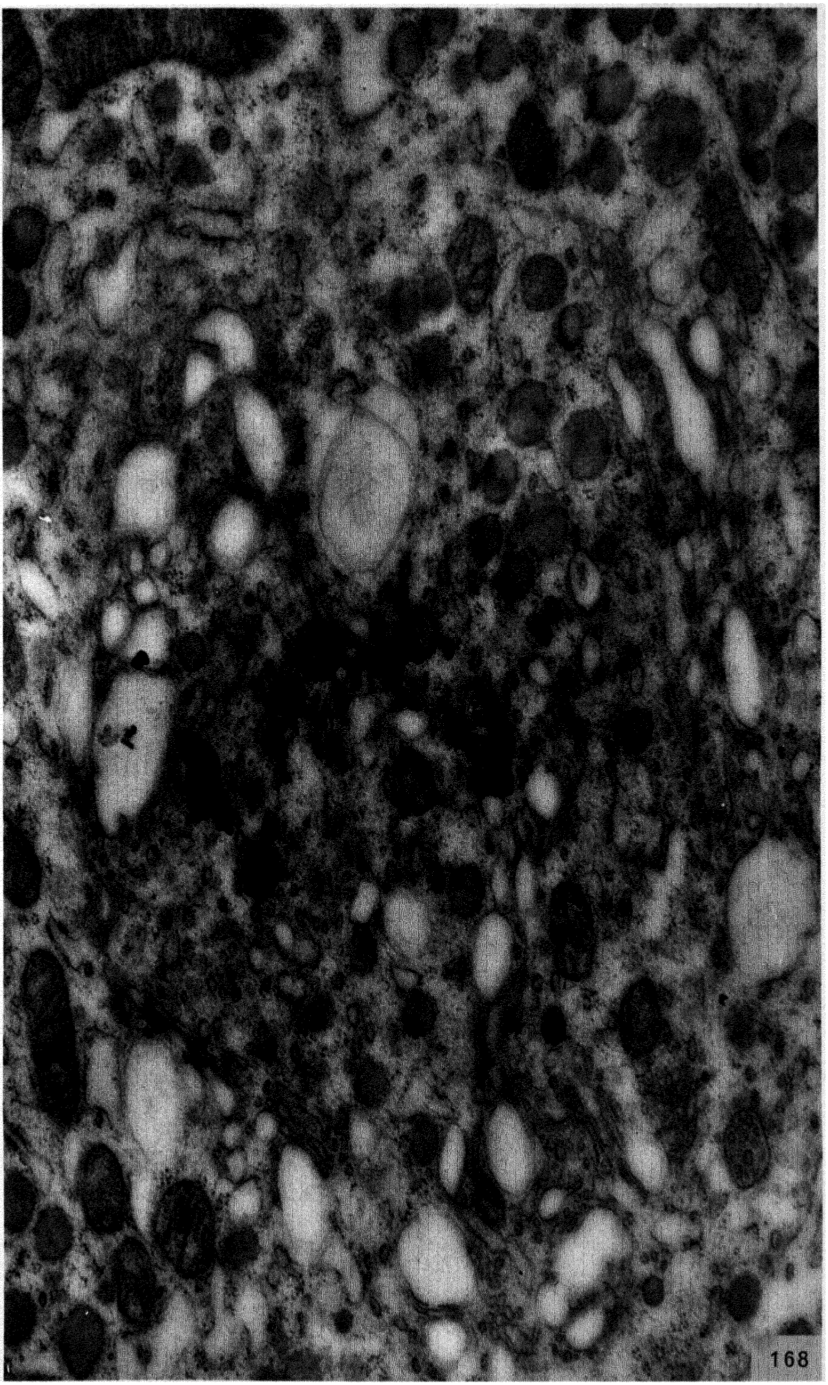
168. Golgi area in the cytoplasm of an acidophile in the adenohypophysis of a normal male rat. Piles of cisternae are arrayed about an area containing swarms of vesicles and secretory droplets in various stages of development. Some of the cisternae are widely dilated. × 32,100.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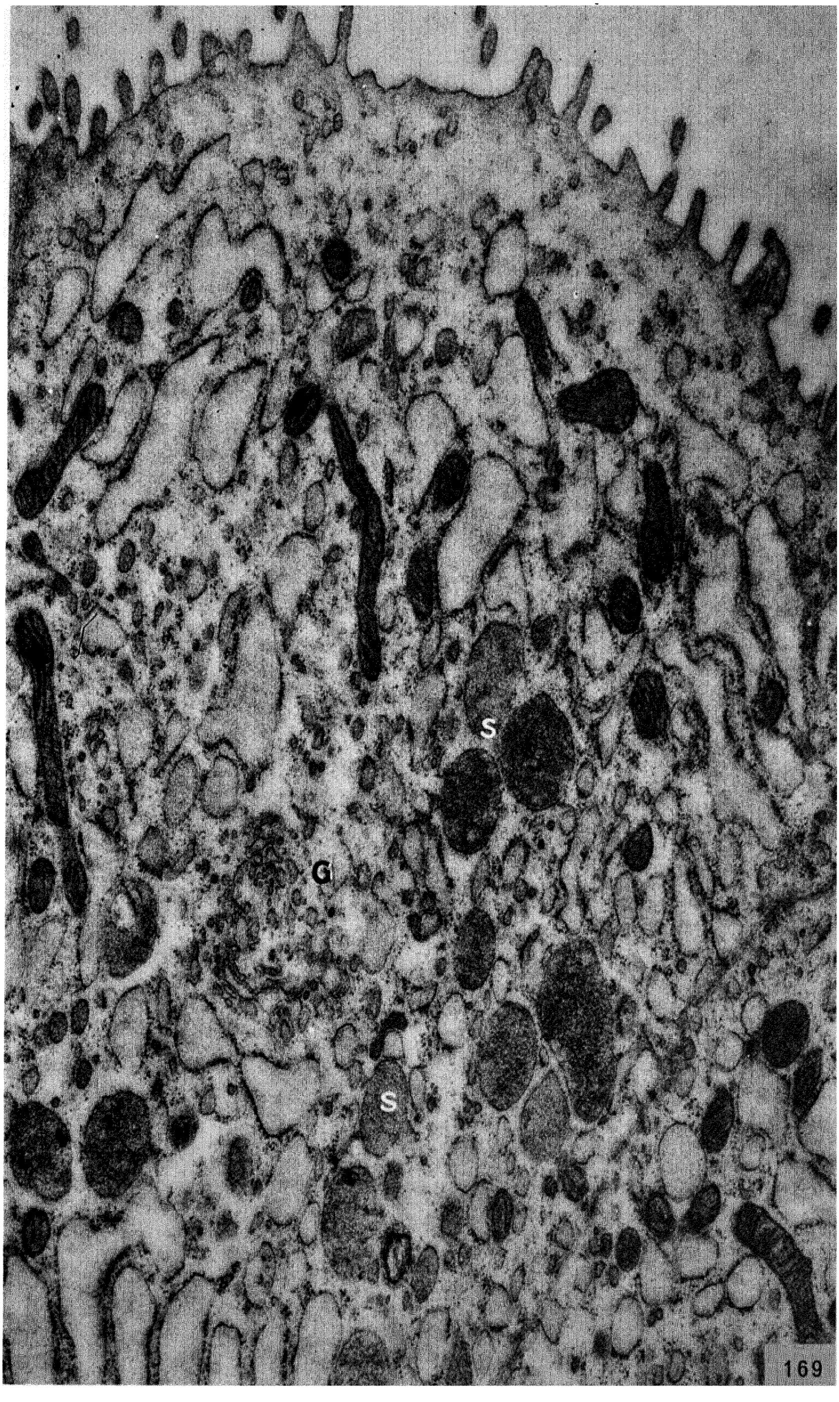
169. Apex of a thyroid follicular cell from a normal male rat. The apical surface is surmounted by irregularly spaced microvilli. The relatively clear cytoplasmic zone just beneath contains a few minute vacuoles. The granular, membrane-bound sacs of the ergastoplasm are dilated, and at the bottom of the figure they are arranged in rouleaux. A small mass of agranular reticulum comprises the Golgi complex (G). Colloid droplets (S) of various sizes and densities are dispersed through the cytoplasm. The mitochondria are usually long, slender filaments. (Micrograph, courtesy of Dr. S. L. Wissig.) × 28,100.



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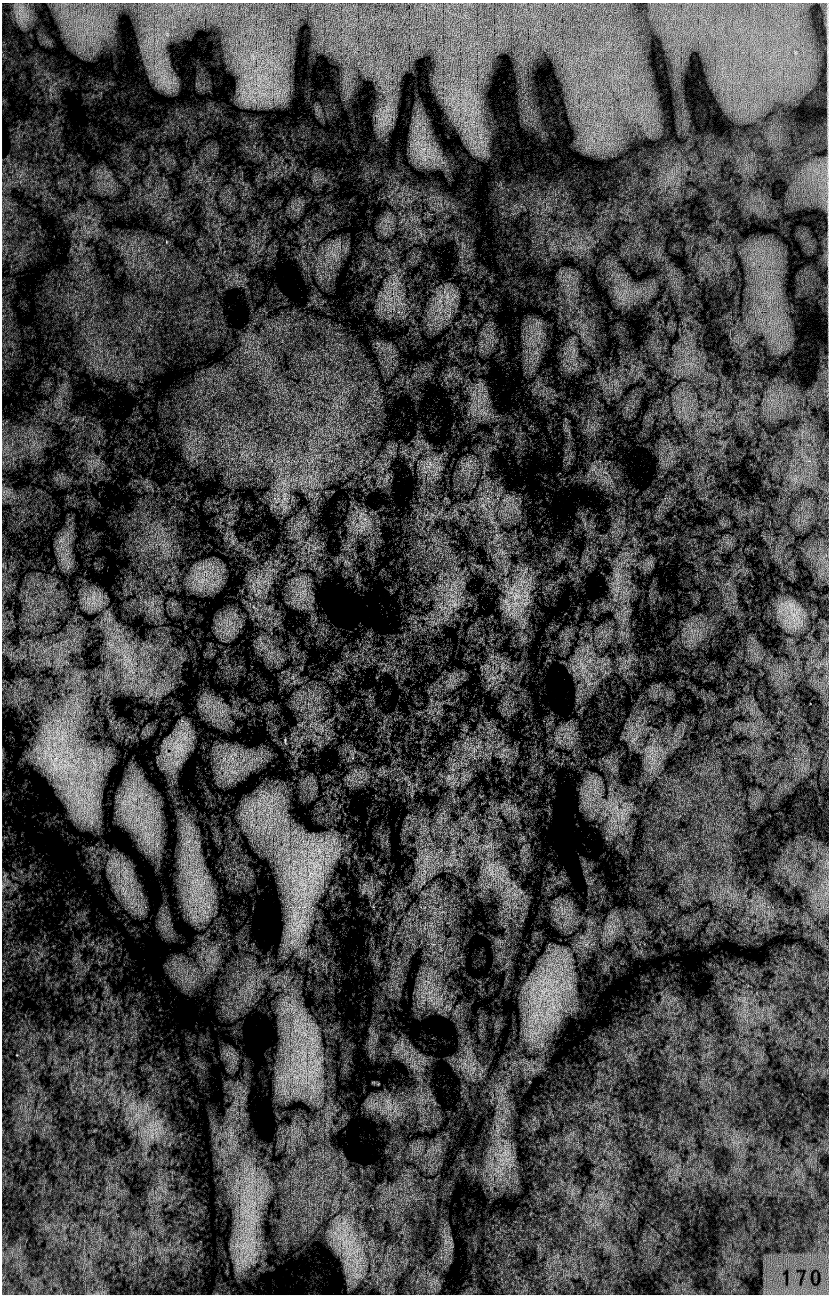
170. Apical portions of two neighboring thyroid follicular cells from a rat that received daily subcutaneous injections of 0.25 per cent sodium hydroxide at a dosage level of 1 ml per 100 gm body weight for 4 days (control for propylthiouracil treatment). These cells display the effects of slight stimulation. The Golgi complex is hypertrophied in both cells. Numerous colloid droplets of various sizes can be seen and intermediate stages in the development of mature, definitive colloid droplets from the Golgi vesicles can be found. The microvilli are taller than they are in the normal cell. Note the numerous small subapical vacuoles. Portions of the nuclei lie in each lower corner of the figure. (Micrograph, courtesy of Dr. S. L. Wissig.) × 28,100.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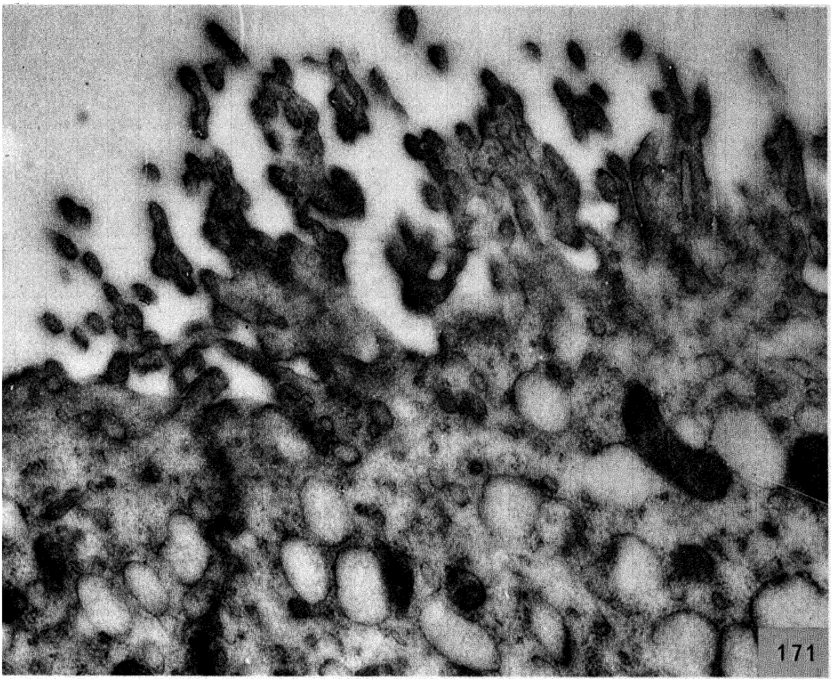
171. Apex of a thyroid follicular cell from a rat that received daily subcutaneous injections of 10 mg of propylthiouracil per 200 gm body weight for 8 days. The microvilli are tortuous and elongated. Their substance is permeated by numerous vacuoles that form a labyrinth in the surface of the cell. Beneath the surface are numerous pinocytotic vesicles which coalesce to form the larger vesicles towards the bottom of the figure. (Micrograph, courtesy of Dr. S. L. Wissig.) × 31,100.

172. Basal cytoplasm of a thyroid follicular cell from a rat that received daily subcutaneous injections of 10 mg of propylthiouracil per 200 gm body weight for 8 days. The nucleus lies at the upper left of the figure. In the center of the figure is an extremely large, branching, ergastoplasmic sac, limited by a single membrane and studded with nucleoprotein granules. The thin cytoplasmic septa protruding into its lumen contain mitochondria and free nucleoprotein granules in addition to cytoplasmic matrix. Such ergastoplasmic sacs communicate with the intercellular spaces at the bases of the follicular cells. (Micrograph, courtesy of Dr. S. L. Wissig.) × 21,500.



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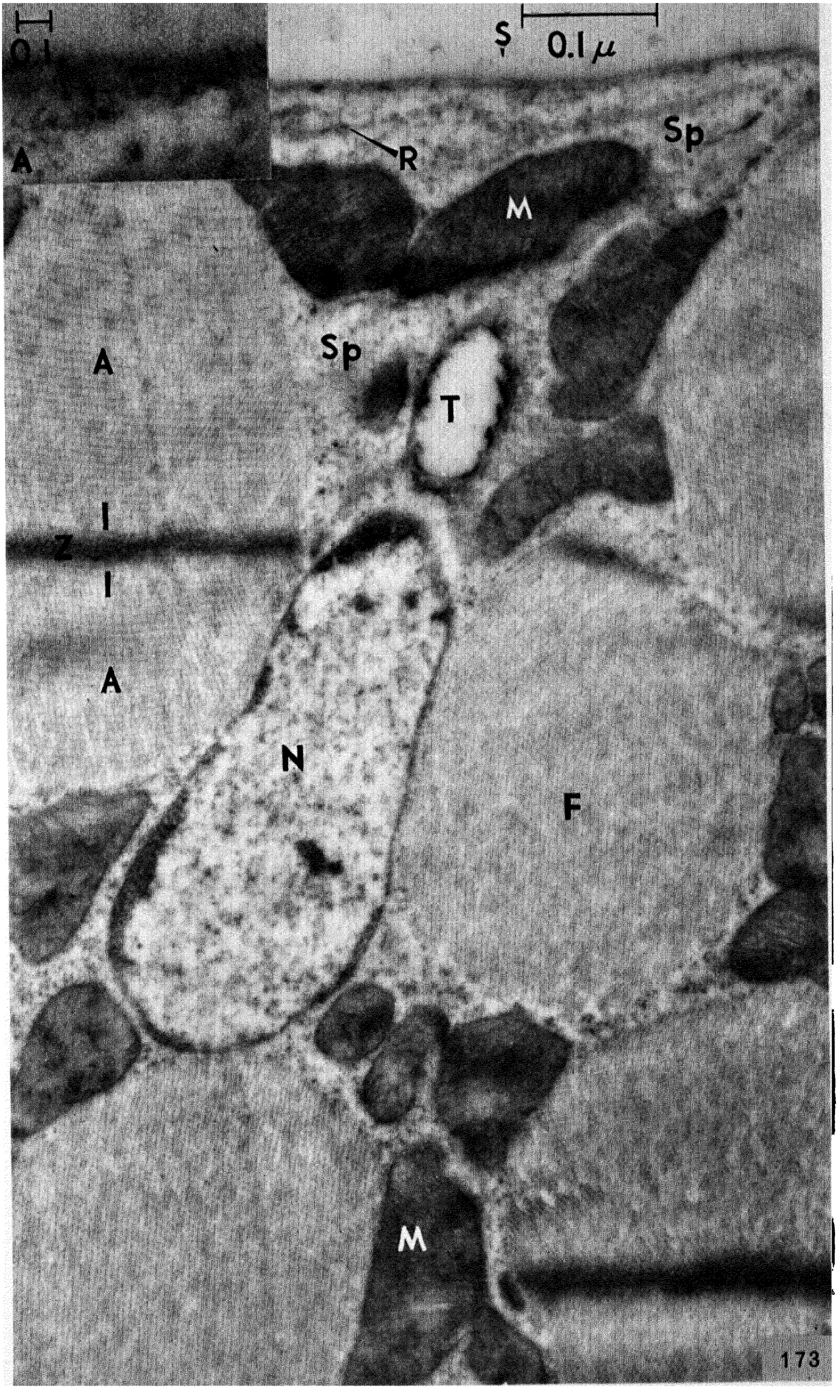
173. Electron micrograph of slightly oblique section through flight muscle of the giant European diving beetle, *Hydrophilus piceus*. The sarcolemma (S) is seen as a fine double membrane across the top. Under it is a pad of sarcoplasm (Sp) containing elements of the sarcoplasmic reticulum (R), mitochondria (M), tracheoles (T), and a nucleus (N). Portions of five myofibrils (F) are shown. In them are myofilaments cut obliquely, connected in hexagonal array by transverse filaments, in confirmation of Hodge [65] (see also Text Fig. A and Fig. 183). The Z band appears in oblique section in several of the myofibrils. A, I, and Z bands can be recognized. × 18,000.

173A. Insert in upper left corner. Detail of sarcolemma, showing two dense layers, each about 200 A thick, between which is a less dense interlamina about 600 A thick. No connective tissue fibers are associated with the sarcolemma in this insect muscle. × 50,000.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174. Drawings of sarcolemma from the paper by Chlopkow [31]. Circumferential markings are shown on the inner surface of the sarcolemma at the levels of the Z bands. These were thought to be fibers by Chlopkow. They were seen in 1875 by Melland, and were noted in electron micrographs by Draper and Hodge [35]. It is now known that these markings represent the sites of attachment of the sarcoplasmic reticulum to the inner surface of the sarcolemma.

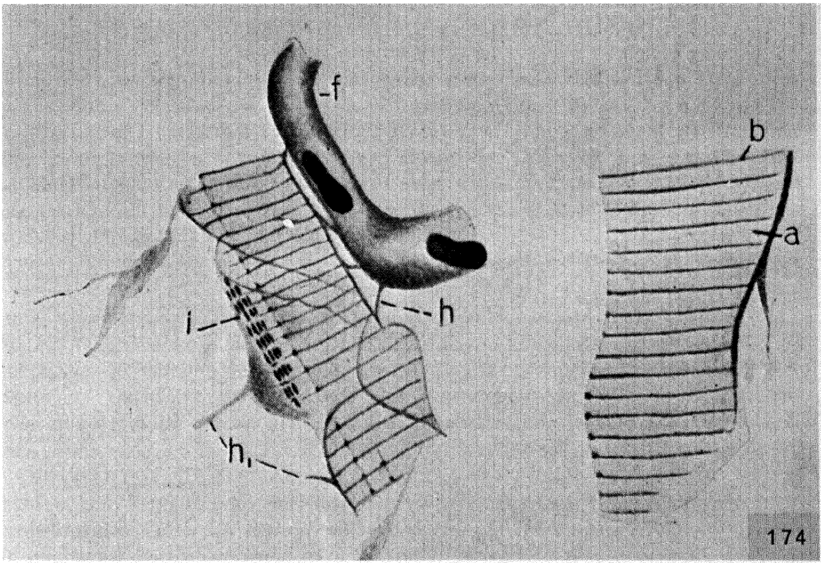
175. One of Bowman's [20] figures showing the sarcolemma of cod fish muscle peeled away from the contractile portion of the muscle fiber. This demonstrates that there is no firm mechanical attachment between myofibrils and sarcolemma. The sarcolemma is structureless at this resolution.

176. Von Boga's [19] representation of a muscle fiber of a larval insect, *Goera pilosa*, showing myotendinal junction. A continuity of myo- and tendinal fibrils is evident. The sarcolemma on the right is separated from the myofibrils by a pad of sarcoplasm bridged by strands of sarcoplasmic reticulum at the levels of Z bands.

177. A figure from Retzius [115], depicting a myofibril from the beetle, *Dytiscus marginalis*. The preparation was impregnated with gold, and shows a delicate longitudinal strand of the sarcoplasmic reticulum running parallel to the fibril, entering into close relationship with mitochondria or sarcosomes (SS), and connecting at one point to a Z band.

178. Another of Bowman's [20] figures of fish muscle, showing myotendinal junctions. Bowman recognized a continuity between myo- and tendinal fibers, but did not conceive of the sarcolemma as enclosing the end of the fiber.

179. A figure of Dobie [34], showing myofibrils of a skate teased apart and displaying delicate stretched strands of sarcoplasmic material extending between the parted fibrils. The sarcoplasmic elements are banded in relation to the cross bands of the myofibrils. This is believed to be the earliest representation of a component of the sarcoplasm which is organized in relation to the cross bands.



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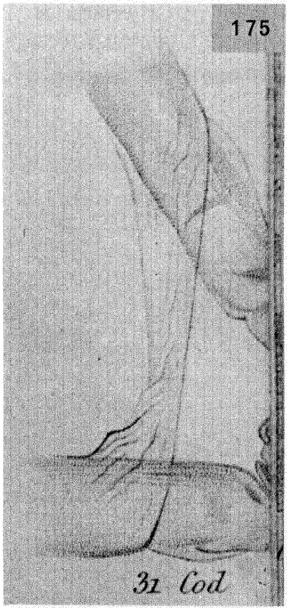

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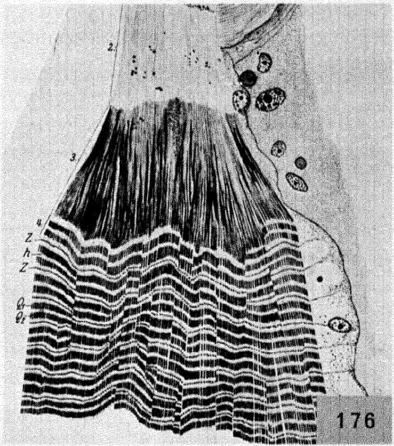

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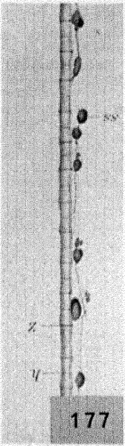

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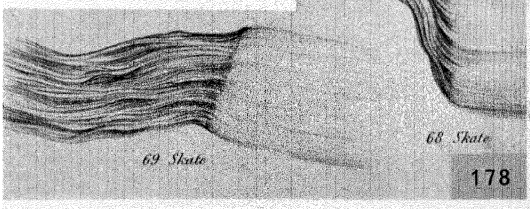

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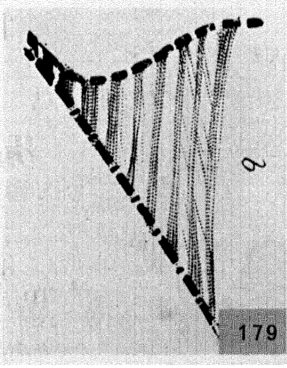
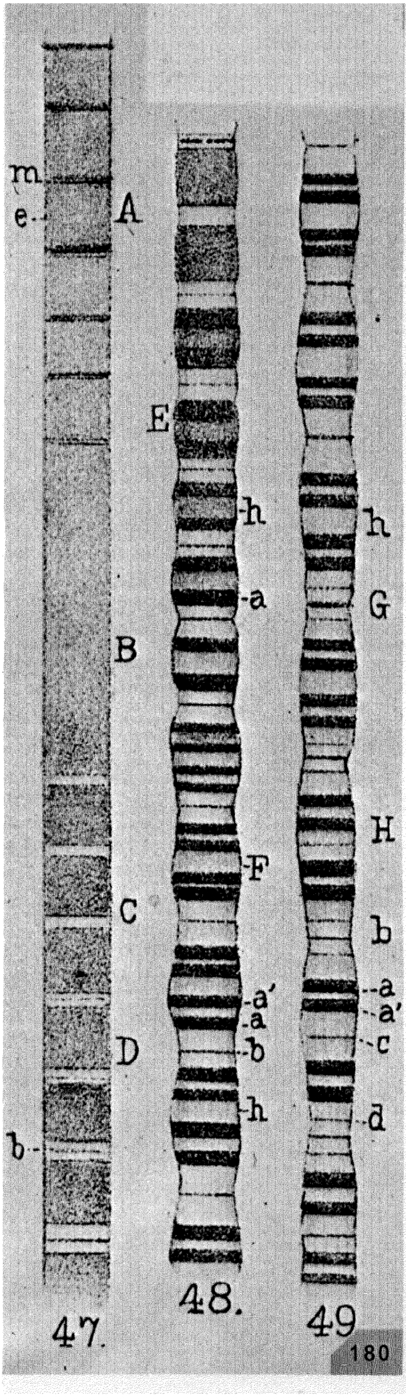


179

180. Cajal's [28] representation of a myofibril from wing muscle of *Hydrophilus*, showing various cross banding patterns encountered in sequence along a single myofibril. The banding pattern in region A at the top of the segment to the left (47) shows sarcomeres with typical contraction bands. The central portion of this segment (B) is one with very faint or absent cross bands. Bennett and Porter [13] found such regions in myofibrils of bird muscle, and suggested that they might represent a stage in the relaxation process. Regions C and D depict cross banding found in relaxed, unstretched sarcomeres, whereas regions F, G, and H show sarcomeres stretched progressively.

181. Kölliker's [81] drawings of myofibrils from wing muscle of the beetle, *Melolontha*, which include the first representation and recognition of the presence of very delicate filaments (now called myofilaments) within the myofibril. A delicate longitudinal fibrillar striation is represented throughout the segment on the right (1), and at the frayed out lower end of the segment on the left (2).

182. Engelmann's [38] sketch of a myofibril from the integumental muscle of the beetle, *Telephorus melanurus*. The left half of the fibril is drawn as seen in ordinary light; the right half as it appears in polarized light between crossed polarizers. The upper part of the fibril shows relaxed sarcomeres; the lower part is contracted. The sequence of banding patterns shows that on contraction the birefringent A band does not change position.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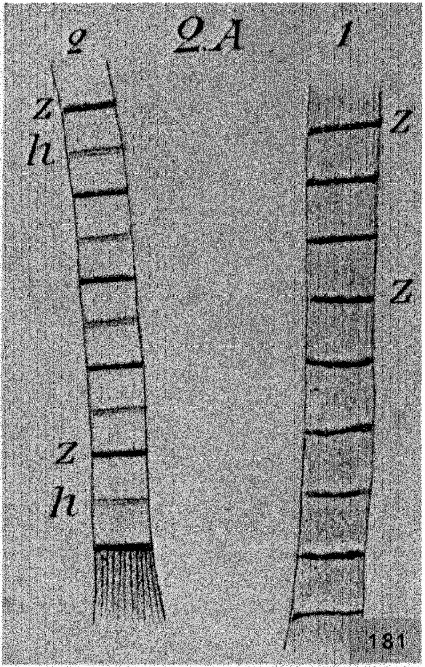
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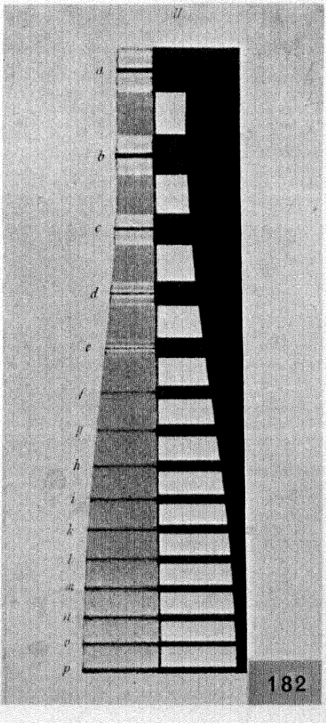
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182

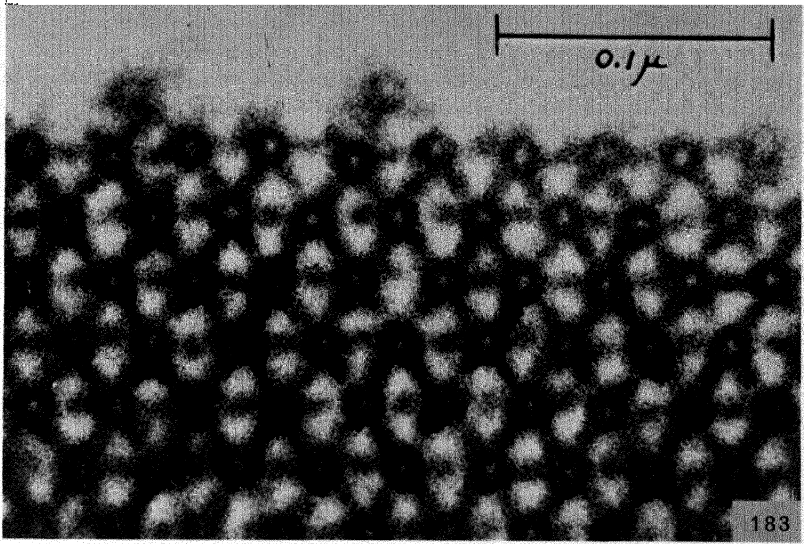
A series of electron micrographs of myofilaments, taken from various authors.

183. Hodge's [65] remarkable electron micrograph of a cross section through the A band of a portion of a myofibril from wing muscle of the blowfly, *Lucilia cuprina*. The myofilaments appear in cross section as dense rings about 120 A in diameter, with less dense centers about 40 A across. These filaments are spaced in hexagonal close packing at distances of 300 A center to center in the fixed muscle, though Hodge states that the separation may be as much as 600 A in the living muscle. Cross-bridging filaments about 40 A in diameter connect the myofilaments transversely, forming equilateral triangles within the hexagonal network. × 330,000.

184. Huxley's [71] electron micrographs of a cross section through the A band of a myofibril from rabbit psoas muscle. This is one of a series of electron micrographs which form the principal evidence for the model of the myofibril proposed by Hanson and Huxley [54]. The micrograph is interpreted by Huxley as showing two sets of myofilaments. One set is comprised of large "myosin" filaments about 110 A in diameter, disposed hexagonally in cross section 200–300 A apart, center to center. Huxley interprets further that his micrograph shows a thin "actin" filament at the center of each equilateral triangle defined at the apices by the myosin filaments. No cross bridges are recognized. × 100,000.

185. A cross section through the A band of flight muscles of the domestic fowl, from the study by Bennett and Porter [13]. Cross sections of the myofilaments appear as round spots demarcating an hexagonal pattern, spaced about 300 A center to center. Here and there one can see dense lines bridging transversely from one filament to another, much as represented in Figure 183, but with less clarity. × 130,000.

186. One of Bennett and Porter's [13] electron micrographs of a longitudinal section through a contracted sarcomere of flight muscle of the fowl. The myofilaments run horizontally and are connected laterally in ladder-like fashion by the cross bridges seen in plane view in Figures 183 and 184. The axial spacing of these bridges is about 170 A in this contracted, fixed, and sectioned specimen. × 120,000.



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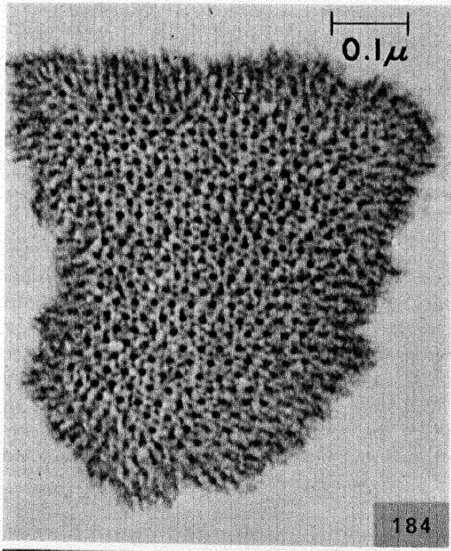

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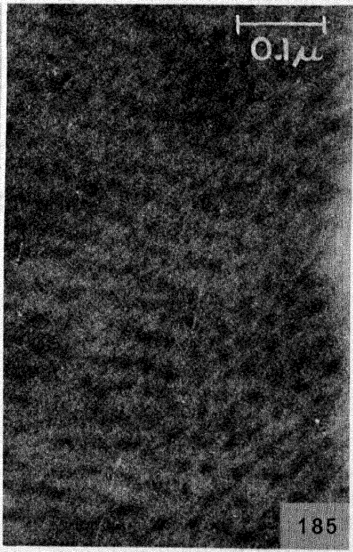

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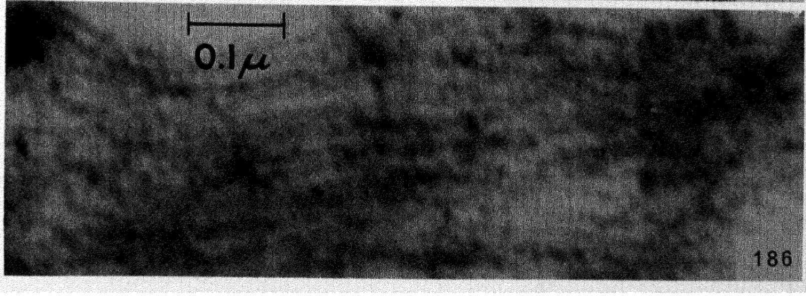

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187. One of Veratti's [137] drawings of the sarcoplasmic reticulum in muscle from a larva of *Gastrophilus equi.* In this variant the reticulum forms a well-defined skein of transverse filaments at the levels of the Z bands, and a less definite transverse plexus at the level of junction of A and I. Irregular longitudinal members are evident. Extensions of the reticulum are shown extending to right and left into sarcoplasmic regions devoid of myofibrils.

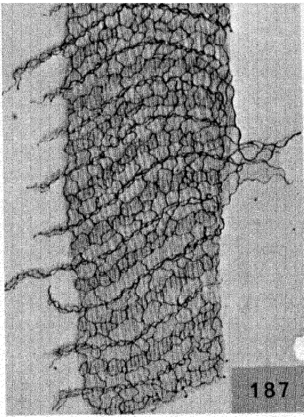
188. Veratti's [137] figure of the reticulum in a muscle from a limb of *Hydrophilus piceus,* impregnated with silver by Golgi's method. In this variant no transverse reticulum at the level of the Z is represented. A plexus of transverse members occur near each junction of A and I.

189. Von Boga's [19] drawing of a muscle insertion in a larva of the Trichopteran, *Goera pilosa.* Continuity of myo- and tendinal fibrils is represented. Extensions of the sarcoplasmic reticulum spray out to the right from Z band levels to traverse in delta fashion the subsarcolemmal sarcoplasmic pad to end on the inner surface of the sarcolemma. Such extensions are not favorably disposed geometrically for the transmission of tension.

190. Veratti's [137] impregnation of the sarcoplasmic reticulum in muscle from the claw of the crayfish, *Astacus fluviatilis.* A well-defined transverse network is evident near the border of A and I, and a more delicate one at the level of the Z.

191. Thanhoffer's [132] drawing of a motor nerve ending from the beetle, *Hydrophilus piceus,* showing an apparent continuity between stained fibrils in the nerve terminal and structures at the levels of Z bands in the muscle—presumably the sarcoplasmic reticulum.

192. Some of Cajal's [28] drawings of the sarcoplasmic reticulum in *Hydrophilus piceus.* Cajal's Figures 1, 2, 3, and 4 are drawn from living limb muscle. Longitudinal elements of the reticulum are shown as straight vertical lines between the myofibrils. The transverse members are represented at the Z lines. Mitochondria or sarcosomes are represented as dots straddling the Z band level between the myofibrils. Cajal's Figures 5 and 6 represent fresh cut transverse surfaces of the living fiber, and show transverse and longitudinal members of the reticulum. Cajal's Figure 19 is of an alcohol-fixed fiber treated with formic acid, but unstained. The characteristic features of the reticulum are evident.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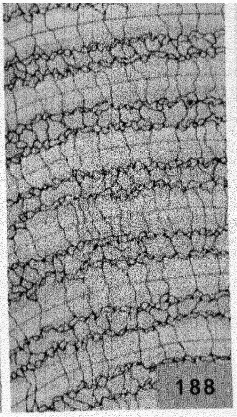
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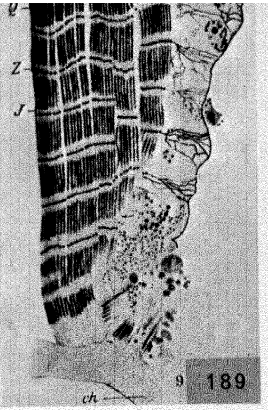

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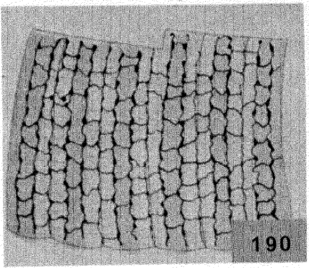
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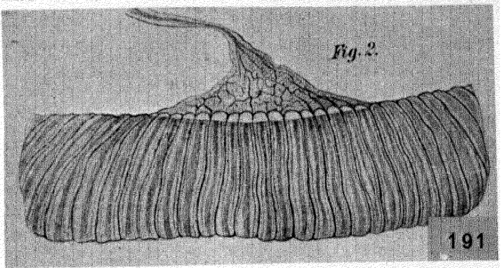

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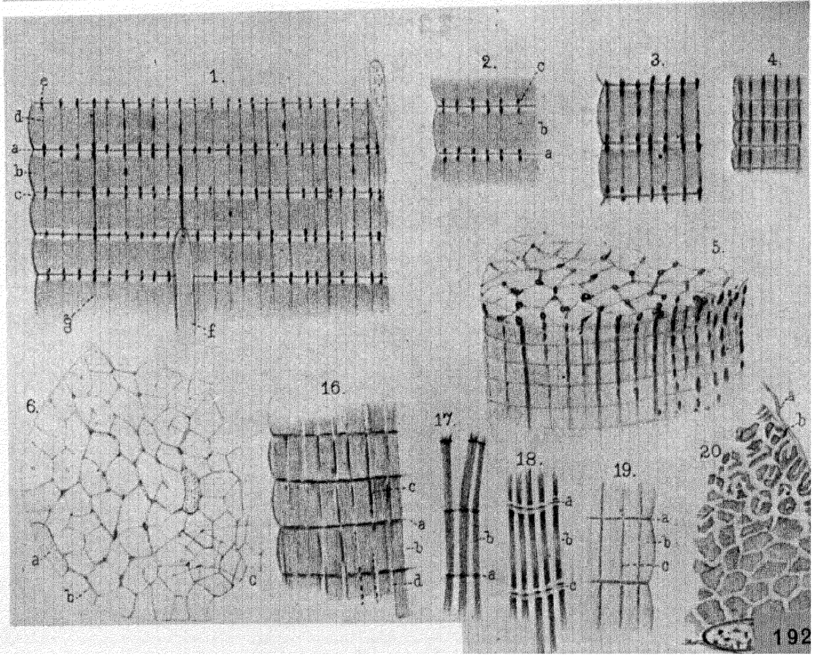

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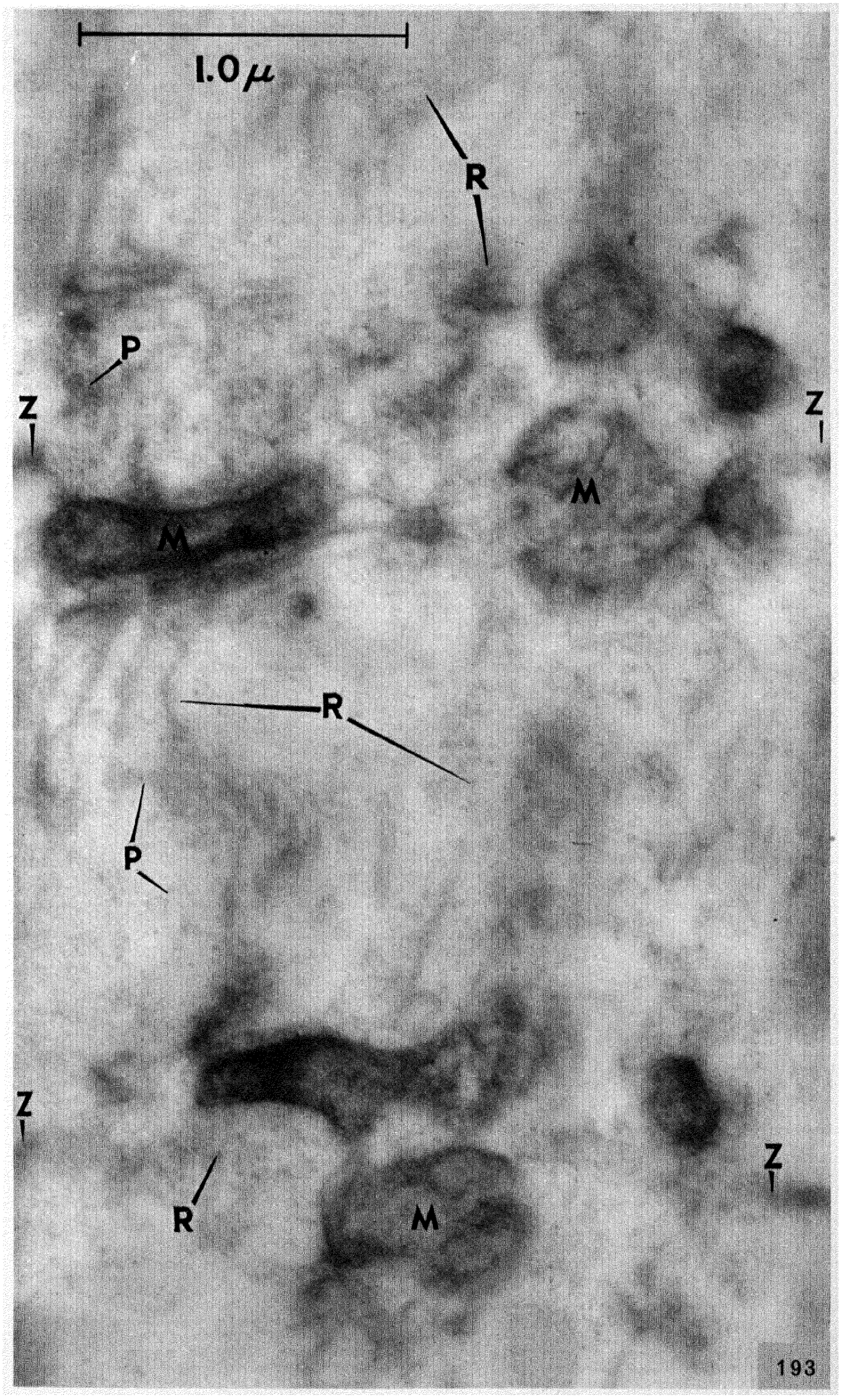
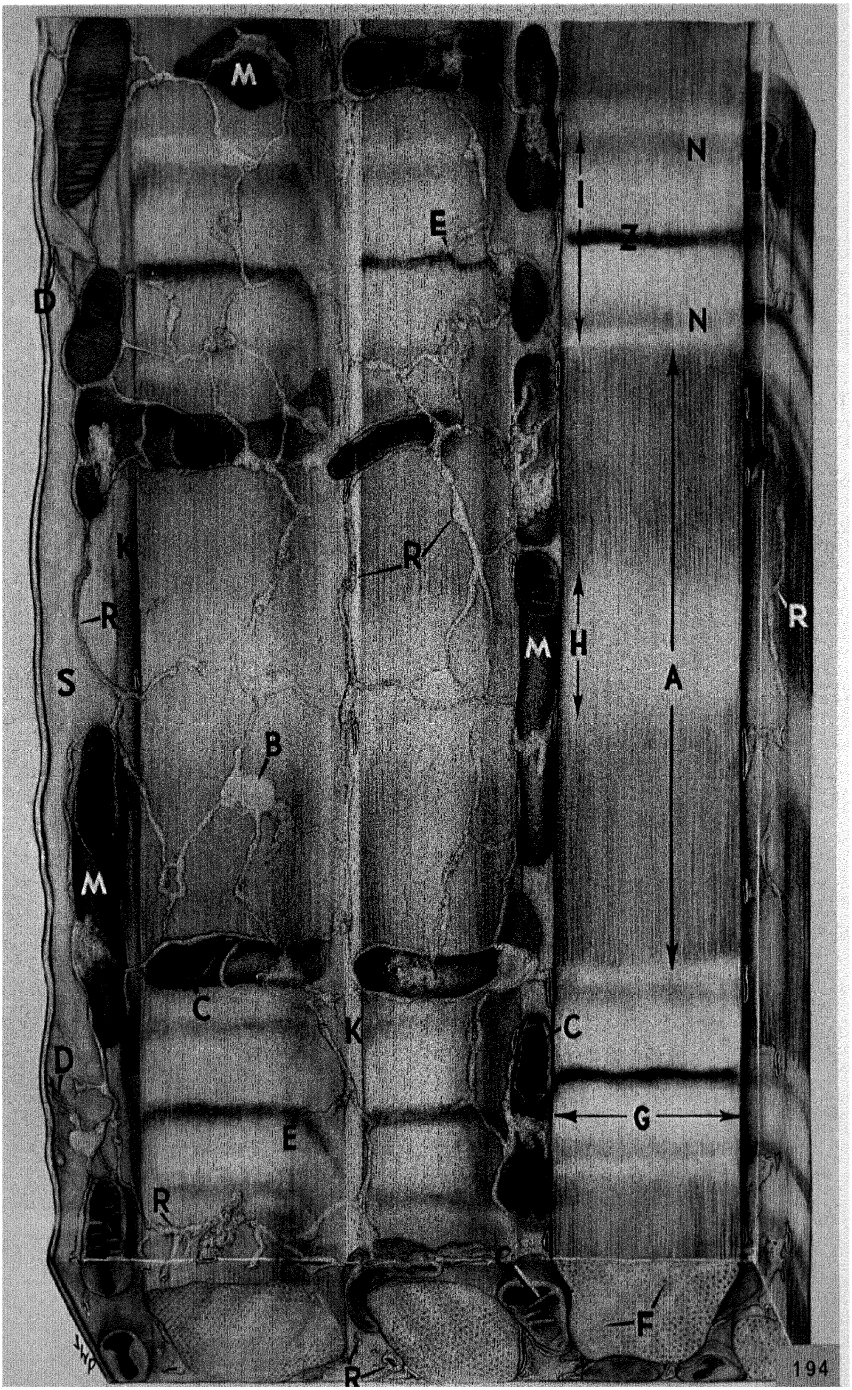
193. An electron micrograph of a section through a portion of the gracilis muscle of a mouse. The section goes through a plane of interfibrillar sarcoplasm. Portions of two myofibrils are included along right and left border of the figure respectively. Two Z bands (Z) can be distinguished in each fibril. A few myofilaments from overlying fibrils can be seen in the upper left corner under the micron mark, and near the lower right corner. The rest of the figure displays sarcoplasm. Portions of several mitochondria (M) are included, arranged preferentially just on each side of the Z band levels. Elements of the sarcoplasmic reticulum are displayed as delicate strands of membrane-like material, adorned with dense particles (P), the particles of Palade [99]. Connections of reticulum with the Z band are seen. Elements of the reticulum are also seen to come into close relationship with mitochondria. In several places the reticulum is cut so as to show oval or bilaminar profiles, betraying a basic tubular and cisternal architecture.



193

194. A schematic model of a small block of striated muscle, about 5 × 2 × 1 μ. The sarcolemma (S) is represented on the left as a membrane bounded on each side by dense sheets. The sarcoplasmic reticulum (R) is represented as a membranous structure forming anastomosing tubes and cisternae (B). On the outside surface of the reticulum are represented the particles of Palade [99]. The reticulum comes close to mitochondria (M), which show internal cristae (C). The reticulum sends strands connecting with the Z bands of myofibrils at E and with the inner surface of the sarcolemma at D. The myofibrils (G) show the standard banding pattern of relaxed muscle, with bands Z, I, N, A, and H designated. The cut ends of the fibrils show myofilaments (F) in hexagonal array. The sarcoplasmic matrix (K) is shown as a structureless medium. After Bennett [17].



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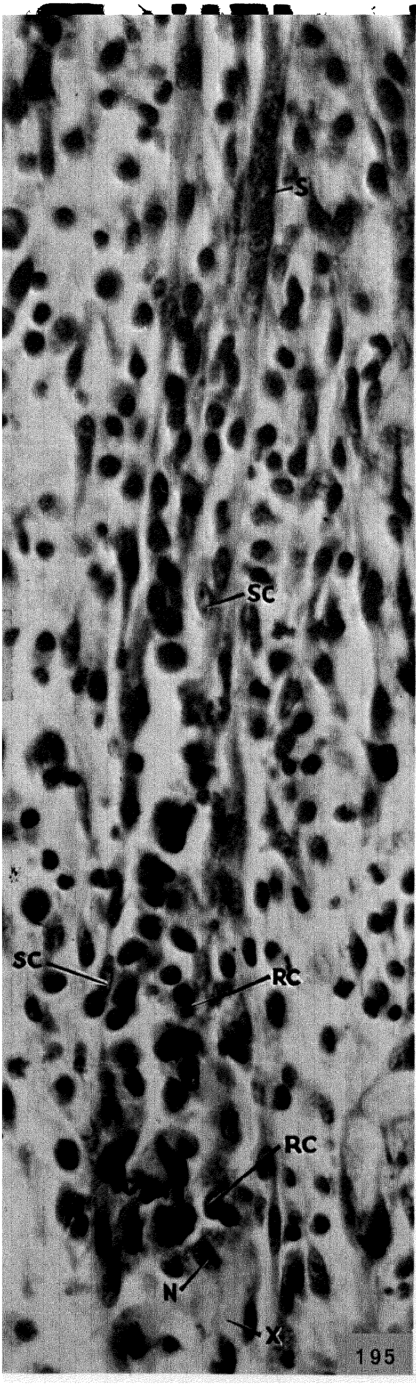
195. Topographic view of a "muscle-cell tube" in border zone between necrotic infarct and surviving muscle, 5 days after ligation of nutrient artery to anterior tibial muscle of rabbit. Partly degenerated muscle masses, some of which are nucleated (N) and show remnants of cross striation (X), beset by round macrophagic cells (RC) which lie toward the expanded distal side. Slender sarcoblastic ribbons (S) are present at the proximal side. Individualized fusiform sarcoblastic cells and straps (SC) are elsewhere applied along the inner aspect of the tube sheath. Hematoxylin-eosin-azure. × 450.

196. Camera lucida drawing of a tube similar to that of Figure 195.



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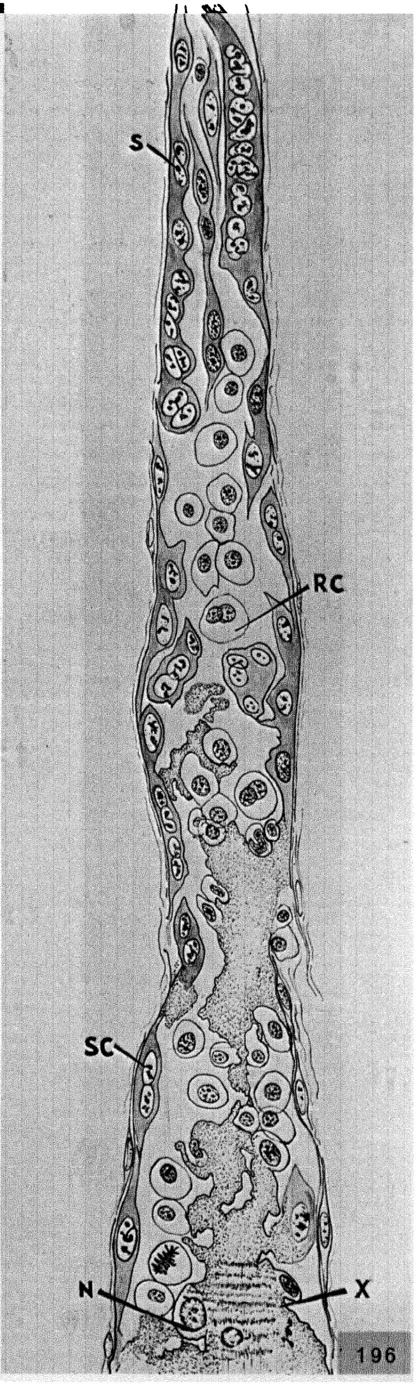

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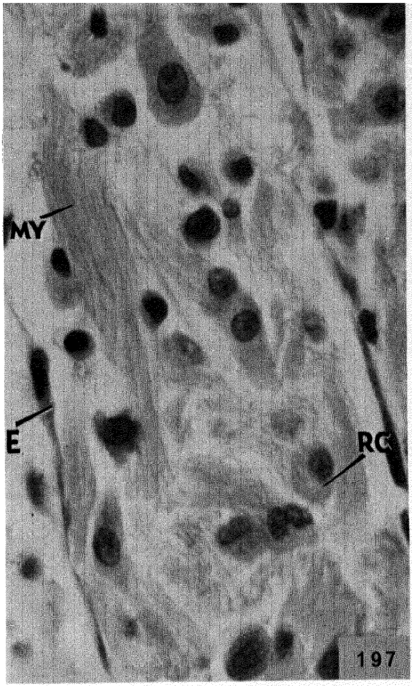
197. "Dissociative" degeneration in a cell-tube containing round cells (RC) in close relation to sheaves of naked myofibrils (MY), some of which are partly included in the cytoplasm of some of the cells. The latter seem to have formed at the expense of viable muscle nuclei and sarcoplasm. E is the endomysial sheath. Hematoxylin-eosin. × 1,000.

198. Cluster of round cells (RC) at the tip of a degenerated muscle fiber segment in rabbit lesion. The cytoplasm of the proximal cells is coextensive with the clotted muscle substance, whose residual cross striation extends into the cytoplasm (X). Single fusiform sarcoblastic cells (SC) lie subjacent to the sheath. Hematoxylin-eosin. × 1,200.

199. Two tubes (A and B) containing the cellular elements encountered in regenerating rabbit muscle. The single round cells (RC) usually lie in tube centers. SB_1 is part of a short sarcoblastic ribbon or strap, and SC are isolated fusiform sarcoblasts lying on the inner aspect of the tube sheath endomysium. SB is a multinucleated muscle "bud." Iron hematoxylin. × 900.

200. Multinucleate giant-cell or bud forms (SB), racquet forms, and ribbon forms of sarcoblast (S), and round cells (RC) in section of outgrowth of tissue culture of rat muscle, 14 days after explantation. Hematoxylin-eosin. × 1,000.



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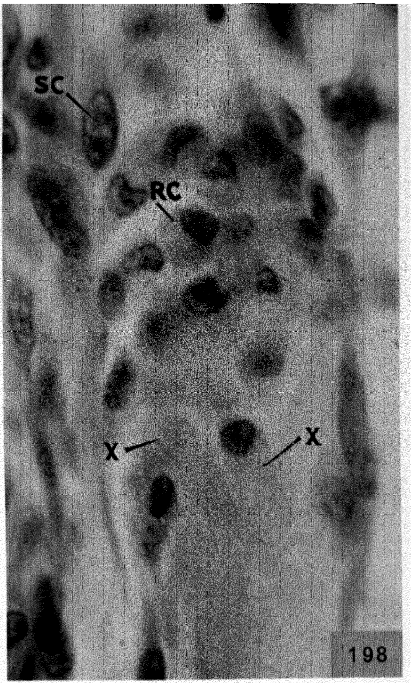

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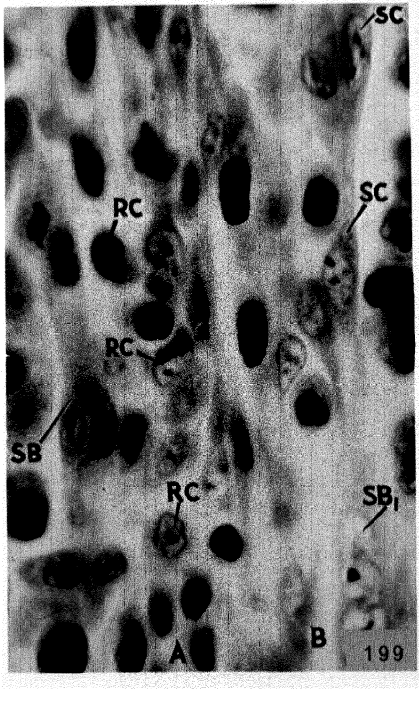

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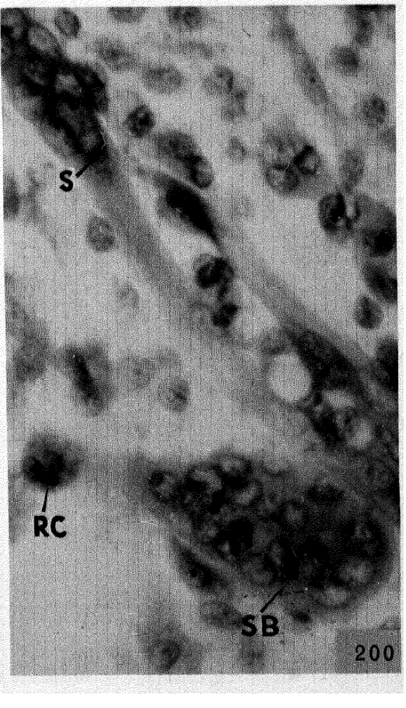

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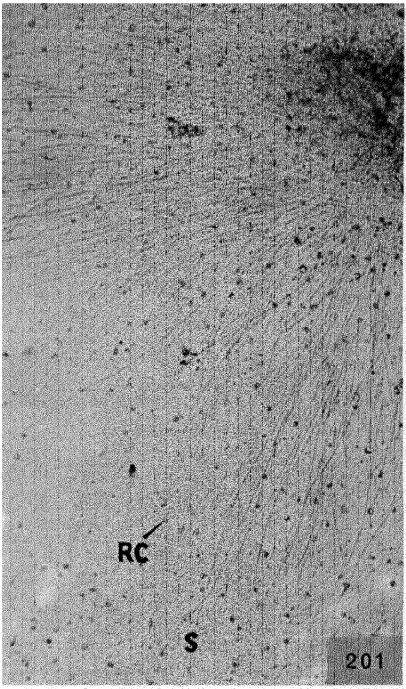
201. Topographic view of part of explant and zone of outgrowth of a tissue culture of rat muscle, 60 days *in vitro*. Long streaming sarcoblastic ribbons (S) and refractile macrophagic round cells (RC) are clearly visible. Living. × 40.

202. Motile macrophagic round cells in upper focal plane of rat muscle culture, where they typically migrate, showing ruffle-like undulating membranes (U), refractile granular cytoplasm, and characteristic forms. Sarcoblastic ribbons and straps (S) are visible underlying them in lower focal plane. Living. × 780.

203. Single macrophagic round-cell (RC) budded from tip of sarcoblastic fibers (S), to which it is attached by a terminal cytoplasmic filament. Tissue culture, rat muscle, living. × 375.

204. Single macrophagic round cell (RC) split out along the course of a sarcoblastic fiber (S). Tissue culture, rat muscle, living. × 375.



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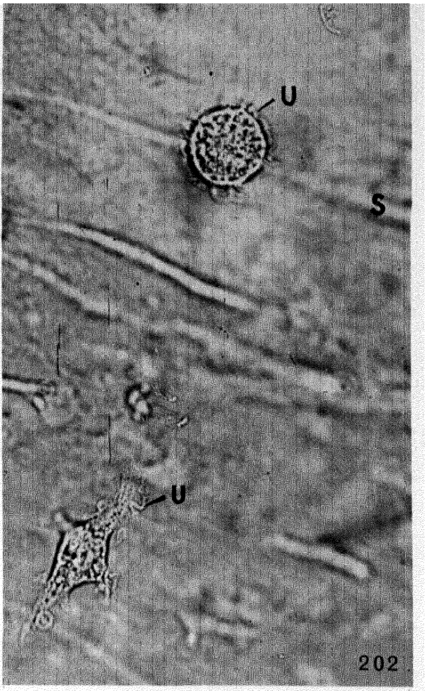

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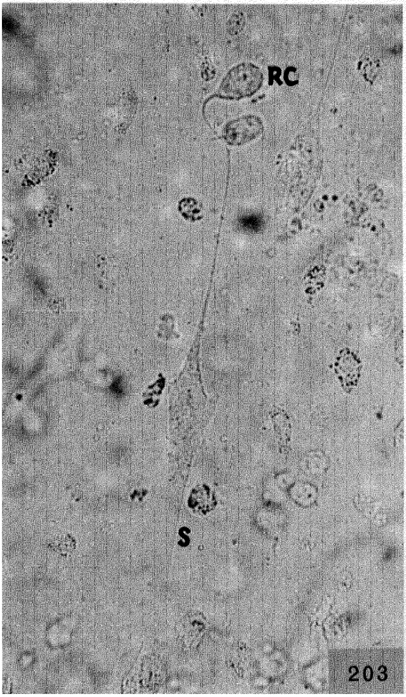

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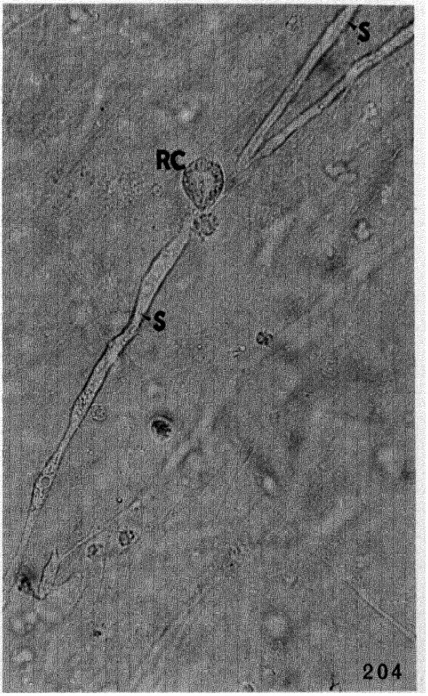

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205. Macrophagic round cells within cell-tube walled by preserved sarcolemma (L) and cleared of necrotic muscle. Cytoplasmic inclusions (I) are evident within the phagocytic cells. Rabbit muscle lesion. Hematoxylin-eosin. × 1,250.

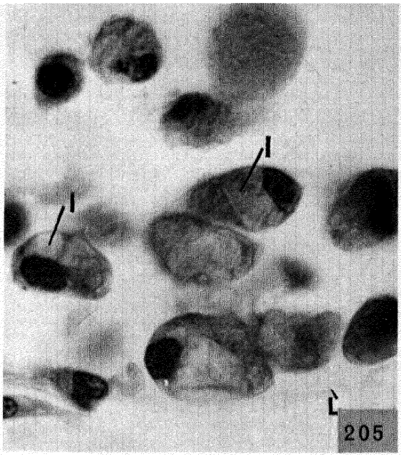
206. Macrophagic round cell (RC) containing a typical rosette of trypan blue (TB), whose cytoplasm is continuous with the substance of the segment of degenerated muscle fiber, from which it seems to have formed. A still viable nucleus (N) is present within the fiber. The nucleus of the round cell is more vesicular than usual, resembling somewhat that of the sarcoblasts, some of which are seen close to another fiber (S). Section of explant of rat muscle, 7 days *in vitro*. Supravital trypan blue, hematoxylin-eosin. × 900.

207. Macrophagic round cells in outgrowth zone of tissue culture of rat muscle showing characteristic rosettes of vital dye (trypan blue) surrounding the large, clear, juxtanuclear cell-center. Supravital trypan blue, carmine counterstain. × 1,250.

208. Polysaccharide-containing material in the juxtanuclear cell-center of a macrophagic round cell in tissue culture of rat muscle. Periodic acid-Schiff stain after diastase digestion and chloroform-methanol extraction. × 1,250.

209. Acid phosphatase activity in juxtanuclear cell center of a macrophagic round cell in tissue culture of rat muscle. Gomori lead method. × 1,350.

210. Distribution of lipide globules and diffuse sudanophilia in cytoplasm of macrophagic round cells in tissue culture of rat muscle. Sudan black B. × 975.



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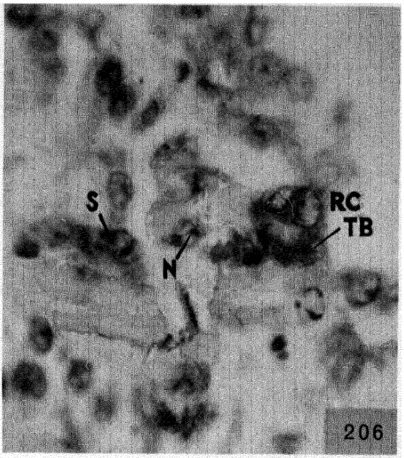

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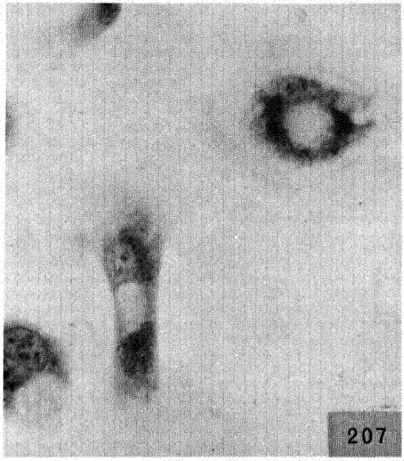

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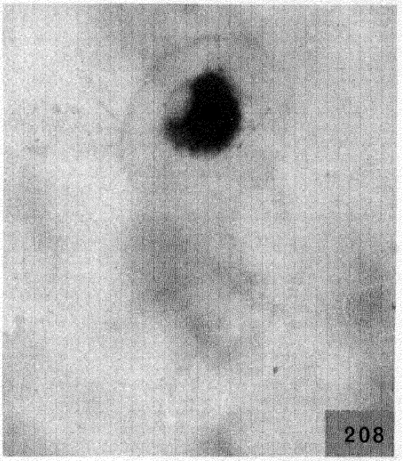

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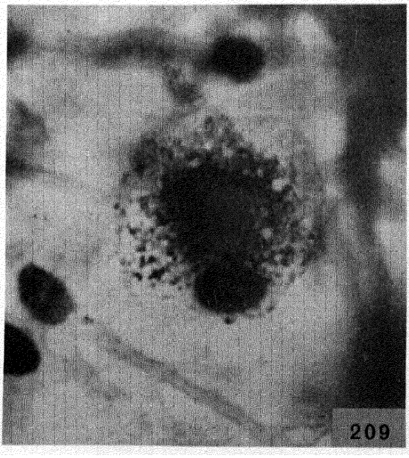

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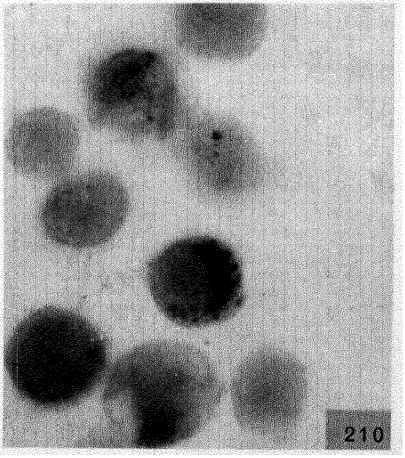


210

211. Sarcoblastic ribbon, prefibril stage, stained to show distribution of ribonucleic acid in cytoplasm and nucleoli. Rabbit muscle lesion, 5th day. Azure B at pH 4.0. × 1,200.

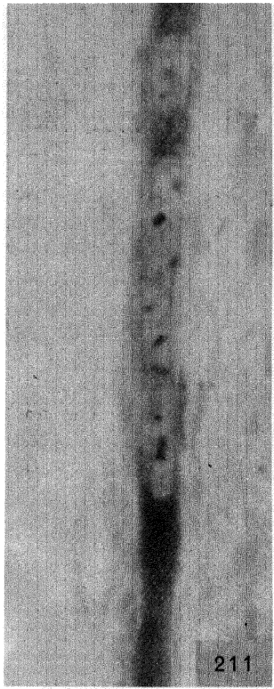
212. Sarcoblastic ribbon, showing earliest traces of fibrillation. The large vesicular nuclei, with their enlarged nucleoli and membrane folds are characteristic. Rabbit muscle lesion, 5th day. Iron hematoxylin. × 1,200.

213. Sarcoblastic ribbon, primarily fibril stage. Rabbit muscle lesion 6th day. Iron hematoxylin. × 1,200.

214. Sarcoblastic ribbon with multiple paraxial fibrils which are undifferentiated. The nuclei lie in an axial column of cytoplasm. Rabbit muscle lesion, 6th day. Iron hematoxylin. × 1,200.

215. Sarcoblastic ribbon with first cross striations consisting of simple alternate banding of the paraxial myofibril. Rabbit muscle lesion, 7th day. Iron hematoxylin. × 1,200.

216. Sarcoblastic ribbon, with first alternate cross striations of multiple myofibrils. This stage is more advanced than that of Figure 215. Rabbit muscle lesion, 7th day. Iron hematoxylin. × 1,200.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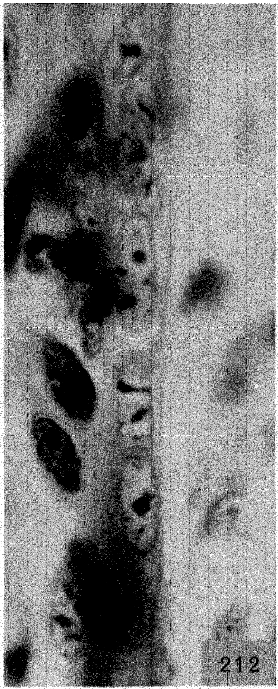
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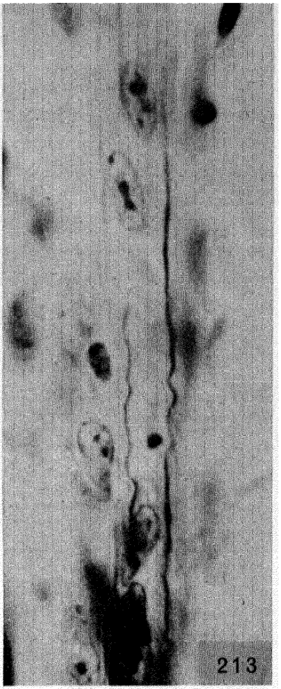
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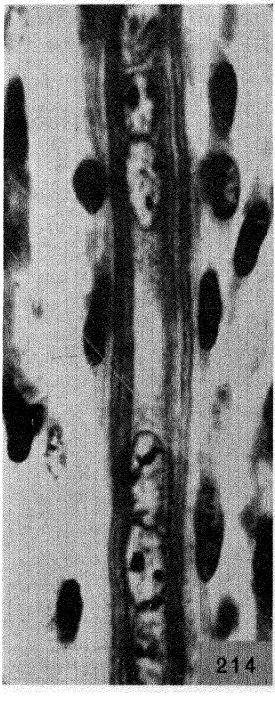
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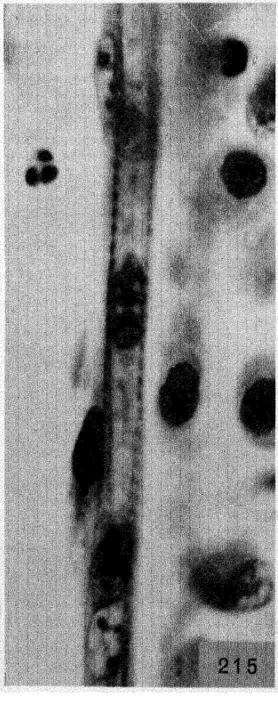
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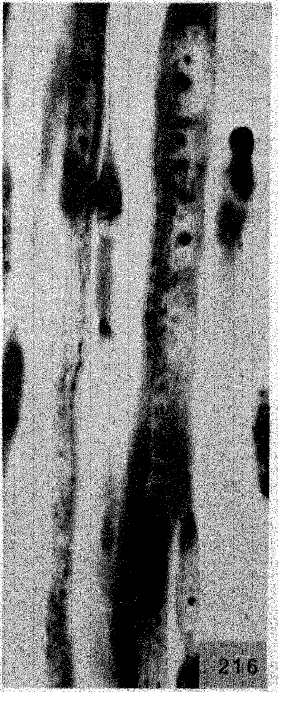

216

217. Adlineation of proliferated sarcolemmic muscle nuclei in muscle fiber preparatory to terminal regenerative outgrowth. Rat muscle, freezing injury. Hematoxylin-eosin. × 750.

218. Characteristic heap or node of contiguous, facetted sarcoblastic nuclei in prefibril sarcoblast. The enlarged rodlike or blocklike nucleoli, the infolded nuclear membranes, and the vesiculated nucleoplasm are noteworthy. Rabbit muscle lesion, 9th day. Iron hematoxylin. × 875.

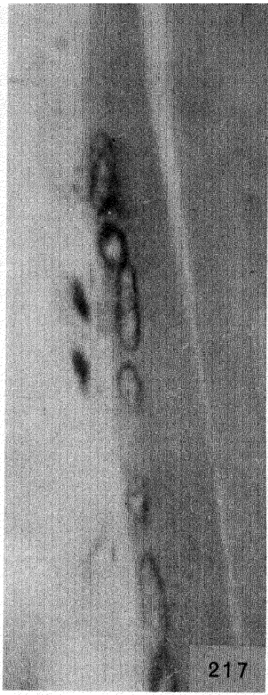
219. Sarcoblastic nuclei of the cross-striate stage, showing aciculate nucleolar inclusions in relation with the nuclear membrane. Rabbit muscle lesion, 9th day. Iron hematoxylin. × 1,200.

220. Nucleus of fibrillated sarcoblast showing incisure of nuclear membrane suggestive of a stage in amitotic division. Tissue culture of rat muscle. Iron hematoxylin. × 1,200.

221. Mitochondria of prefibril or early fibrillated sarcoblast showing oriented longitudinal array. Tissue culture of rat muscle. Pollak silver method. × 1,000.

222. Differentiated fiber in tissue culture of rat muscle, showing full complement of striations, including A discs, M lines, I discs, and Z lines. Iron hematoxylin. × 1,100.

223. Partly matured regenerated fiber, about ½ the diameter of a comparable adult rabbit muscle fiber. The increased numbers and size of the nuclei, and the relatively large amount of sarcoplasm between fibrils are noteworthy features. Rabbit muscle lesion, 14th day. Hematoxylin-eosin. × 1,000.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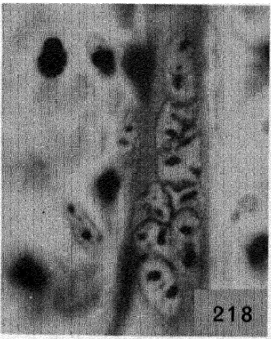
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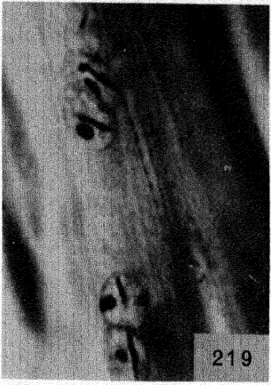
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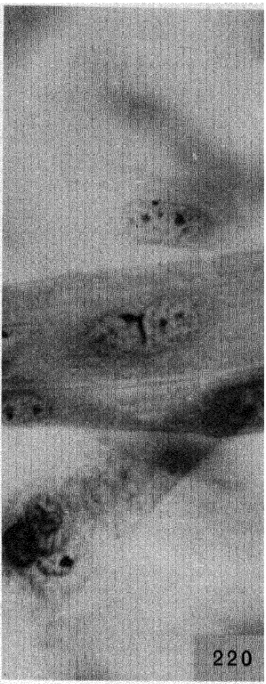
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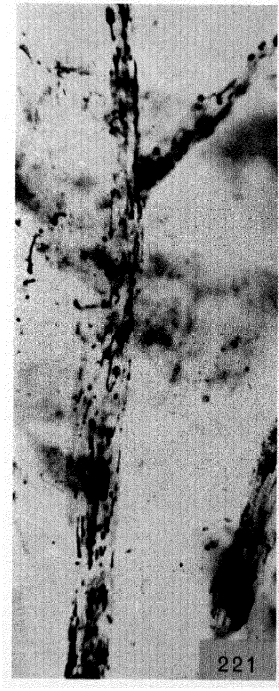
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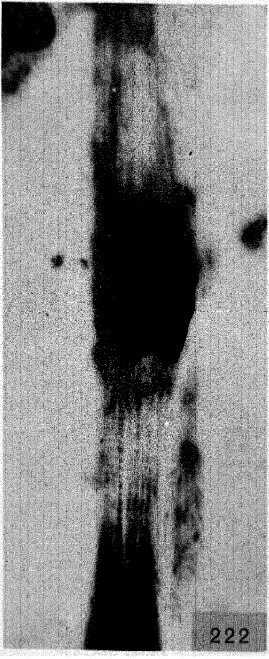
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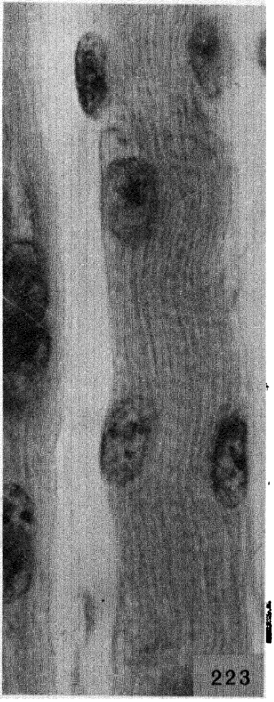
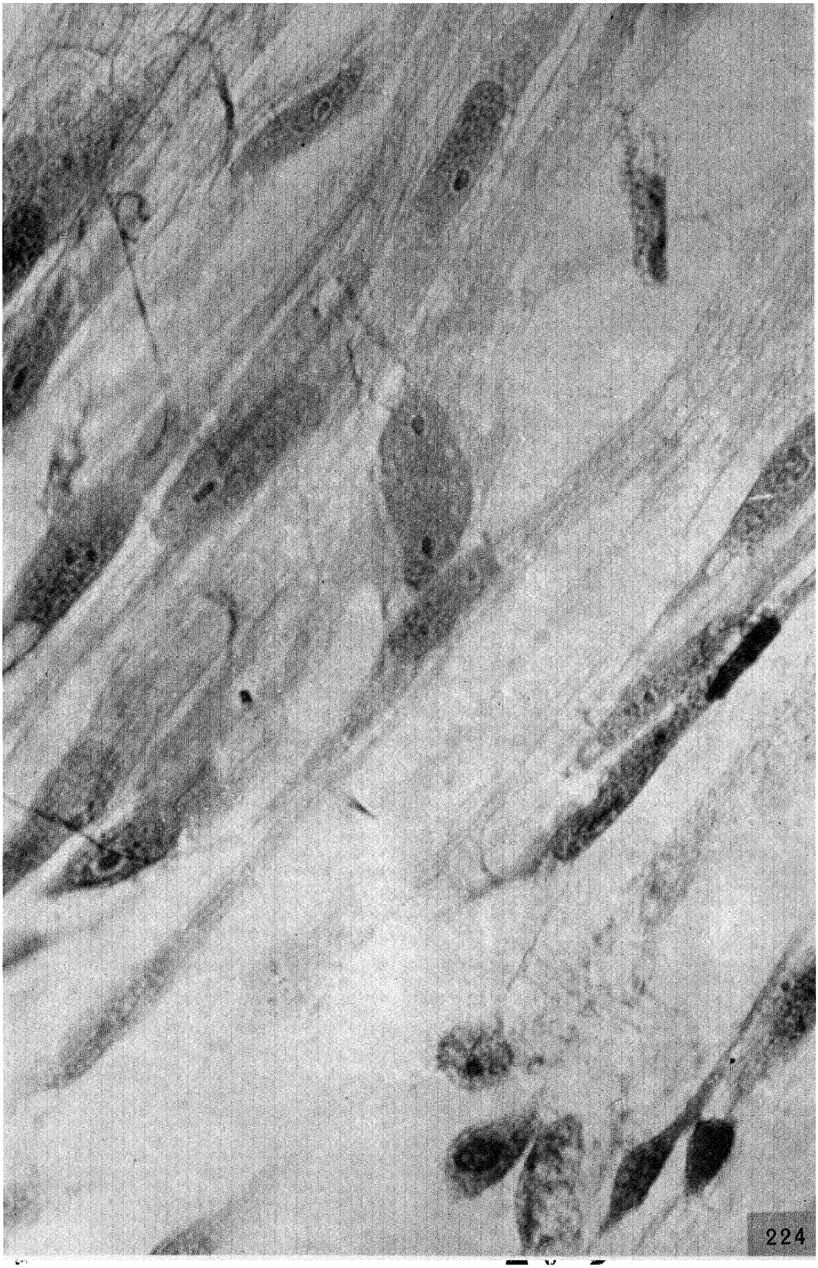

223

All pictures demonstrate tissue cultures of whole fibroblasts. With the exception of Figure 228, all figures are microphotographs of tissue cultures infected with poliomyelitis virus. In Figure 228 the culture of fibroblasts was injured with the supernatant fluid of poliomyelitis-free human stool.

224. As compared with the control, the tonoglia of the fibroblasts infected with poliomyelitis are markedly decreased, thus becoming more visible. In the cytoplasm of some of the fibroblasts vacuoles can be seen. In the lower right corner are some fibroblasts revealing more advanced stages of degeneration. Masson's stain (modification of Pappenheim). × 2,000.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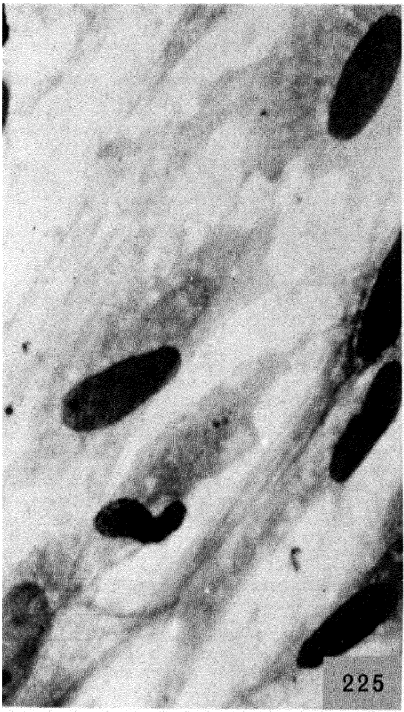
225. Moth-eaten appearance of the cytoplasm of fibroblasts with protoplasmic projections. Notice the vacuolization of the cytoplasm. Giesma stain. × 1,800.

226. Displacement of the nucleus by vacuoles and granules. The nuclei reveal hyperchromatosis. Nuclear debris can be seen. Giesma stain. × 1,800.

227. Marked vacuolization of the cytoplasm. In the lower cell, displacement of the nucleus by a vacuole can be observed. Giesma stain. × 1,800.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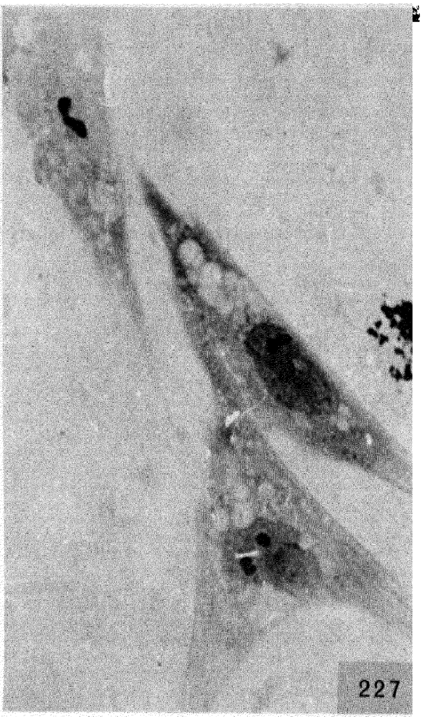
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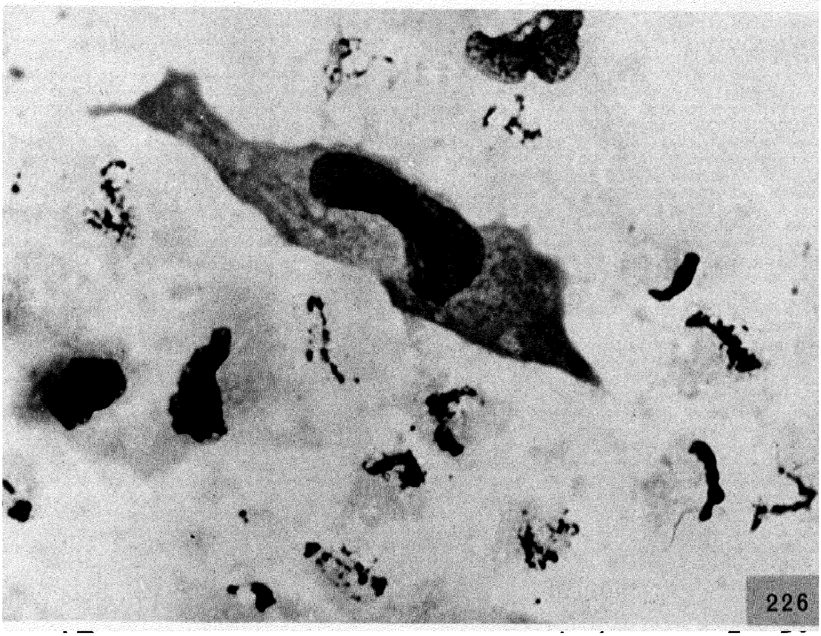
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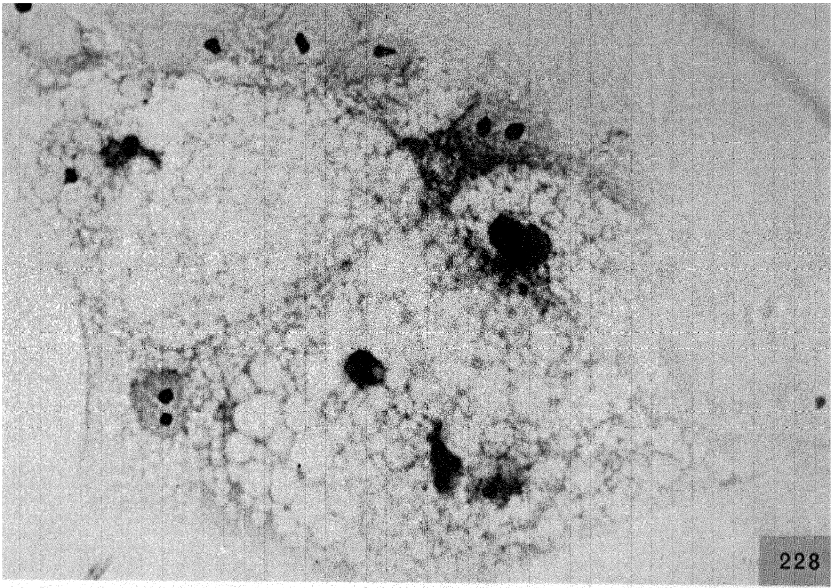
228. Marked vacuolization of fibroblasts injured with the supernatant fluid of poliomyelitis virus-free stool. The vacuoles of such cells were stained pinkish with Nile blue sulphate. Notice that the fibroblasts are not separated from each other. Giesma stain. × 1,800.

229. Vacuoles and granules in cytoplasm of a fibroblast. Giesma stain. × 1,800.

230. Mitotic figure in a fibroblast. Nuclear debris is seen in its vicinity. Giesma stain. × 1,800.



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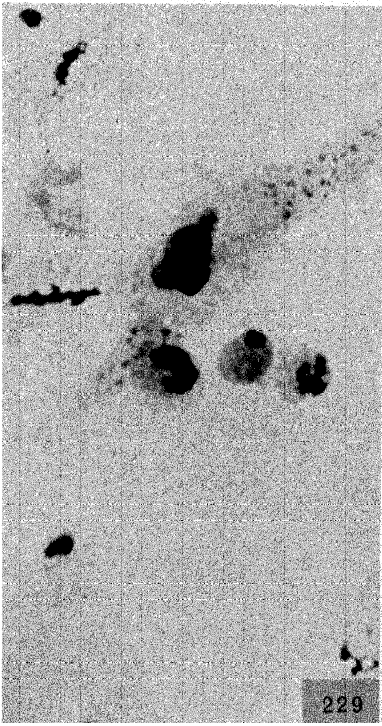

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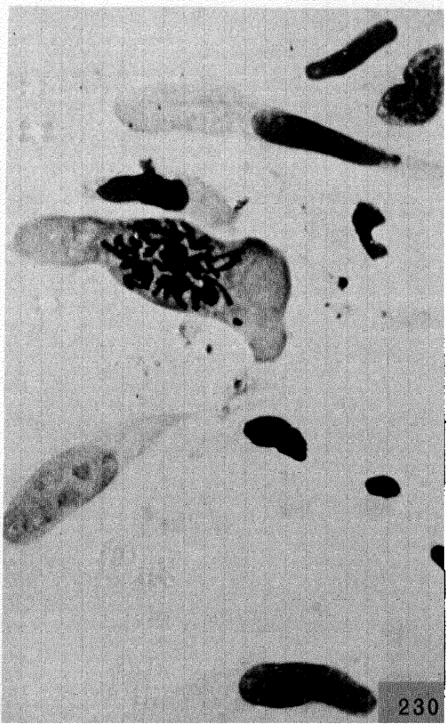
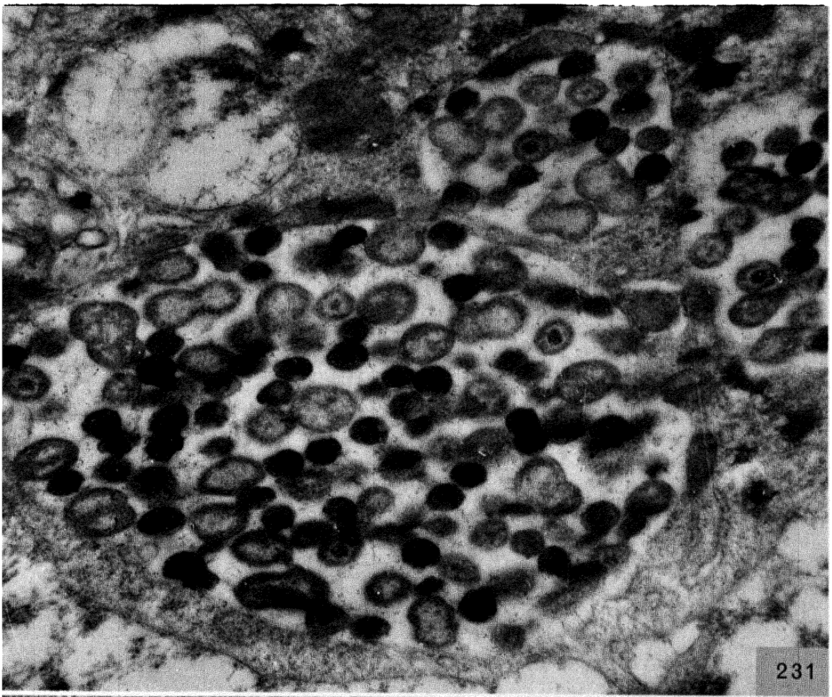


230

231. Meningopneumonitis virus. Unlike "true" viruses this agent seems to multiply by binary fission. The reproduction takes place within vesicles in the cytoplasm. Each vesicle is surrounded by a membrane and viral development is apparently more or less independent of the cellular metabolism [27]. × 17,700.

232. Vaccinia virus. Large "plastin" covered inclusion bodies in the rabbit cornea. These are probably true Guarnieri bodies. Developmental forms of the virus can be seen as a network of small circles in the largest inclusion body [29]. × 6,000.



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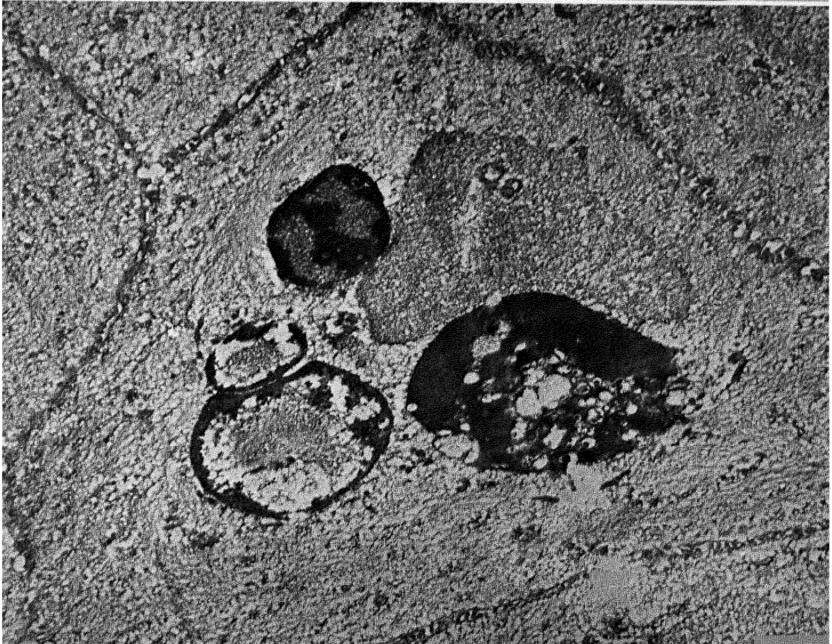
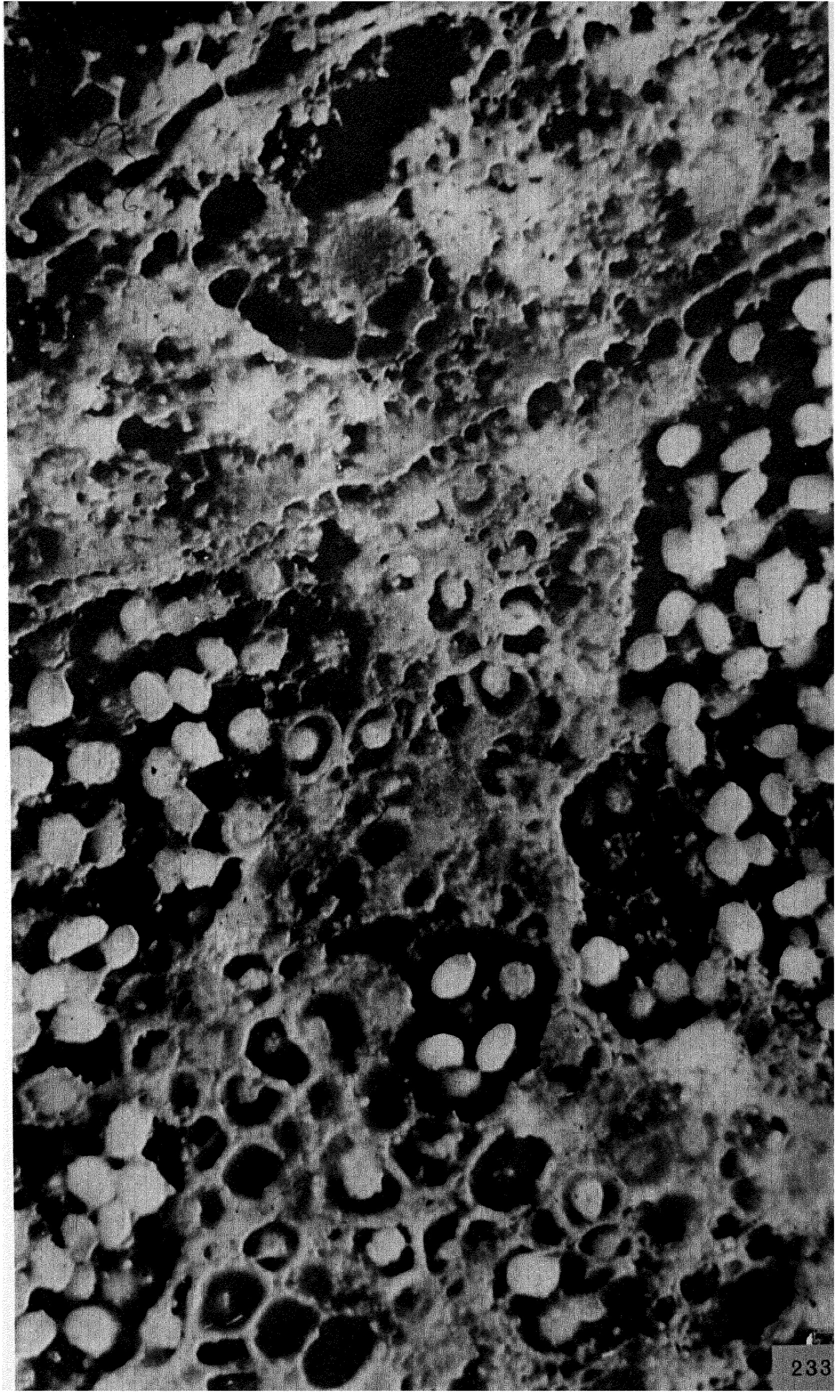


232

233. Molluscum contagiosum virus. Locules of mature virus and intervening septa. The pockets of virus here differ from vesicles (as in Fig. 231) in that they are not surrounded by a membrane. Virus seems to be formed directly from the cytoplasm through a series of circular developmental forms honey-combing the septa [2]. × 27,400.

W. H. Gaylord



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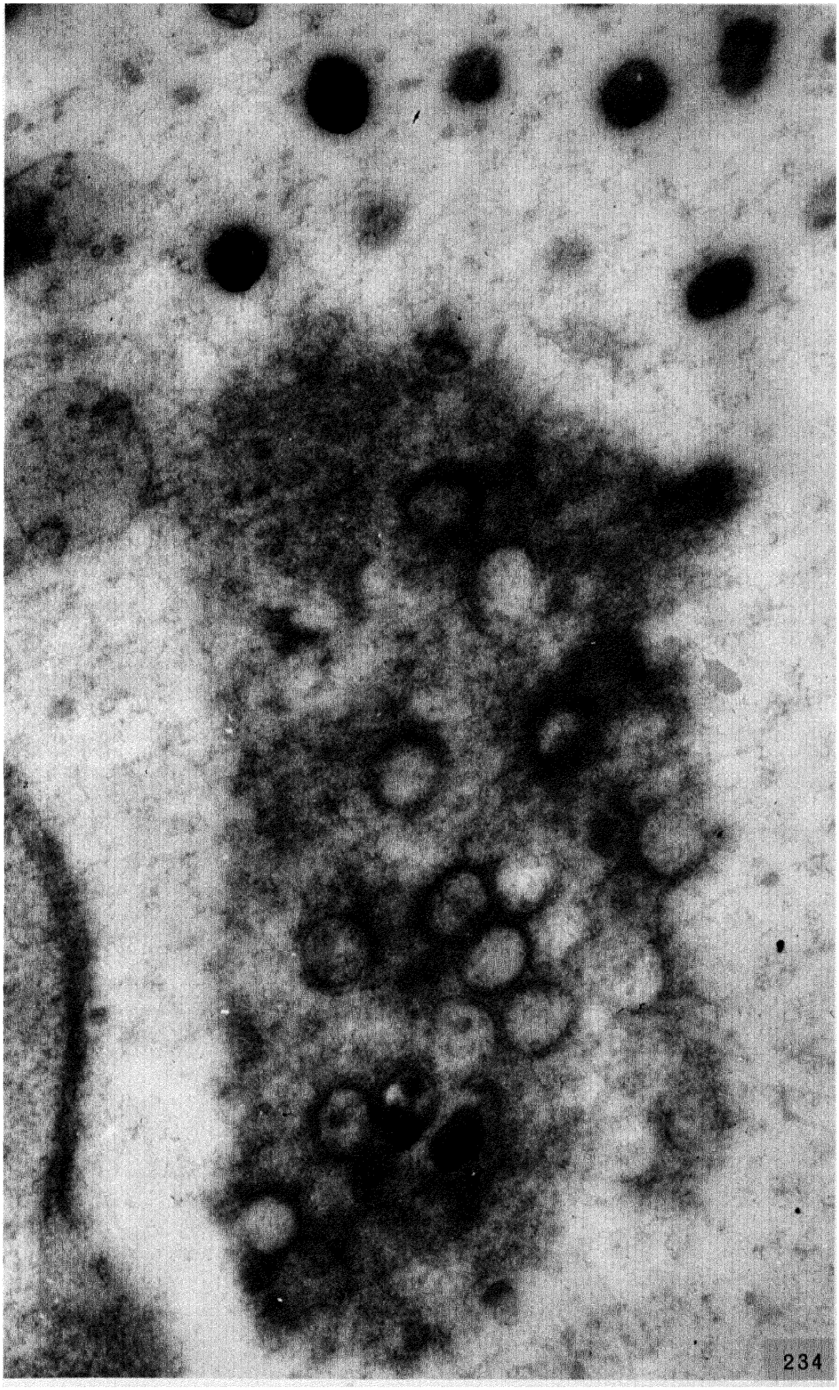
234. Vaccinia virus. Formation of early developmental stages in a cytoplasmic matrix. Circular membranes appear to form directly from the dense material. The developmental forms are analogous to those seen in molluscum contagiosum lesions and are a characteristic stage of all pox viruses. Nuclear membrane at left; dense, mature virus at top. Large, low density, circular structures have not been identified. The dense material obscuring virus development as seen in the cornea (Fig. 232) is absent in cells of the chorio-allantoic membrane [29]. × 33,000.



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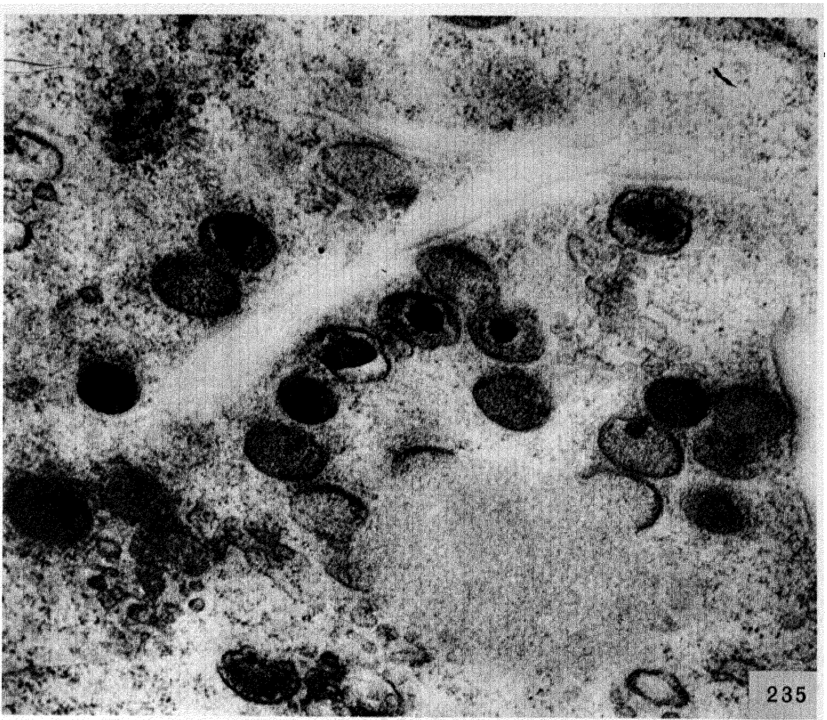
235. Vaccinia virus. Various stages of virus development. Membranes seem to be forming around patches of granular material at lower right. Characteristic "nucleoid" and "viroplasm" are visible in several particles [44]. × 38,000.

236. Vaccinia virus. Extracellular particle. Detail of the fine structure of a mature particle, showing the arrangement of internal membranes. The particle is in contact with the external surface of the cell membrane [44]. × 131,000.



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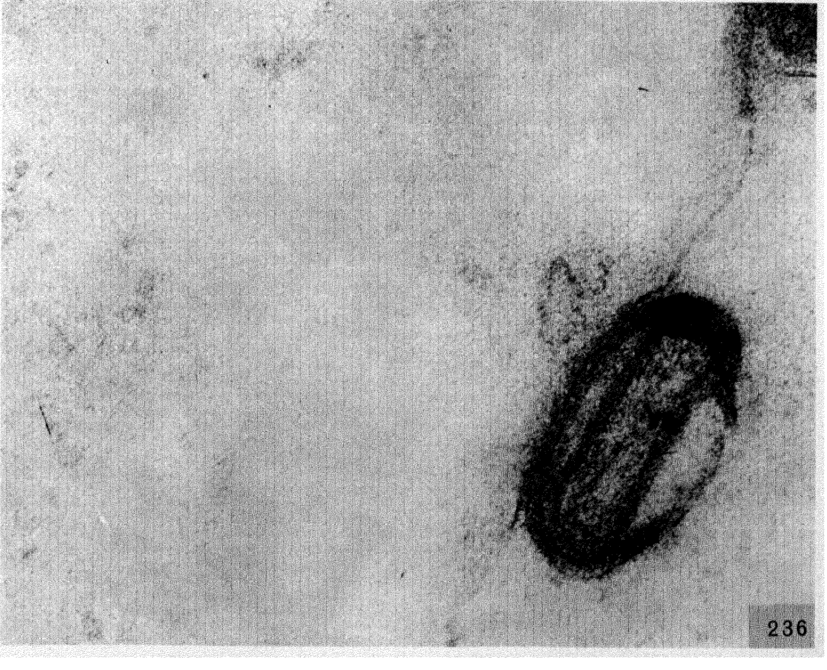
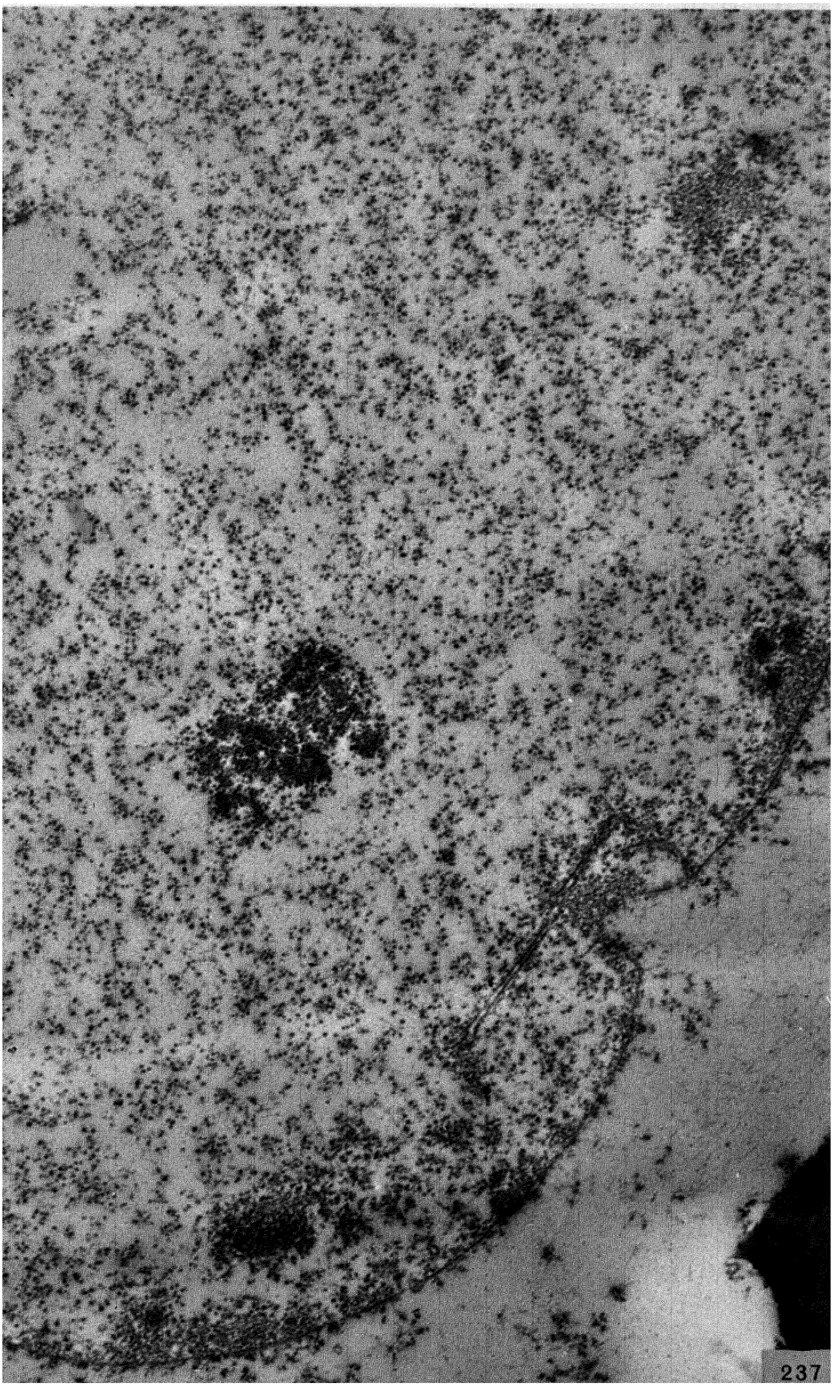


236

237. Virus-like particles associated with human skin papilloma. Diffuse distribution of particles in the nucleus and a central cluster. Nuclear membrane is intact but virus particles can be seen in the cytoplasm. Parallel lines are a result of deep infoldings commonly seen in the nuclei of these cells. Portion of characteristic cytoplasmic mass showing at lower right. This is an early stage of development. (Previously unpublished micrograph by H. Bunting) × approximately 33,000.



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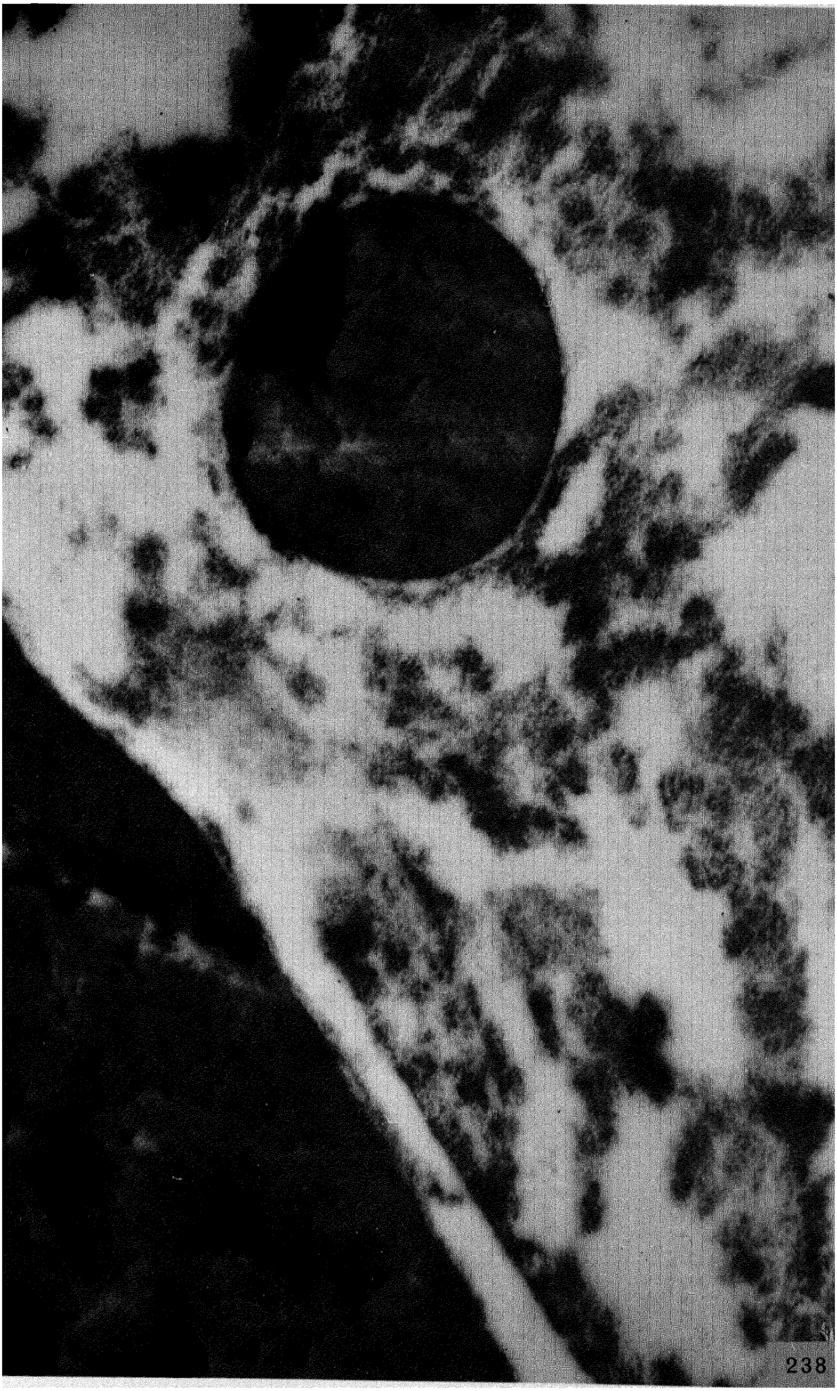
238. Intranuclear inclusion body and portion of a cytoplasmic mass in an infected papilloma cell. The round, circumscribed, eosinophilic inclusion in the nucleus is a typical Cowdry type B. It is indistinguishable from the cytoplasmic mass at lower left. Aggregates of virus particles can be seen almost filling the nucleus. They are not directly associated with the inclusion body, although both inclusions may be obscuring large numbers of virus particles. (Previously unpublished micrograph by H. Bunting) × 22,000.



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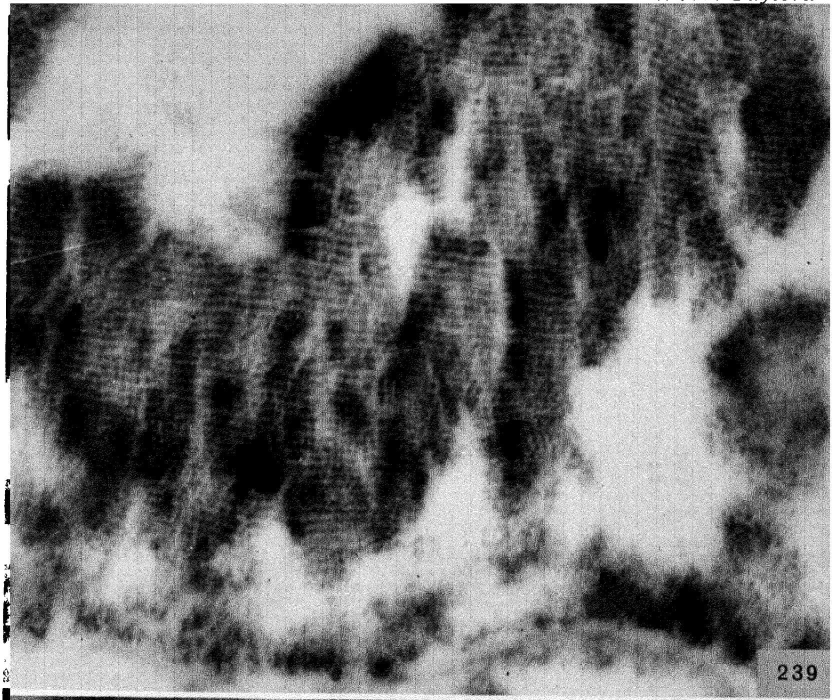
239. Linear array of papilloma virus particles. Nuclear membrane at bottom. At this stage the virus-packed nucleus has started to collapse. Virus particles show characteristic tendency to form a regular pattern [15]. × 33,700.

240. Hexagonal close-pack of papilloma particles. The nucleus and all other recognizable features of the cell are gone and only a crystalline arrangement of virus particles remains [15]. × 32,000.



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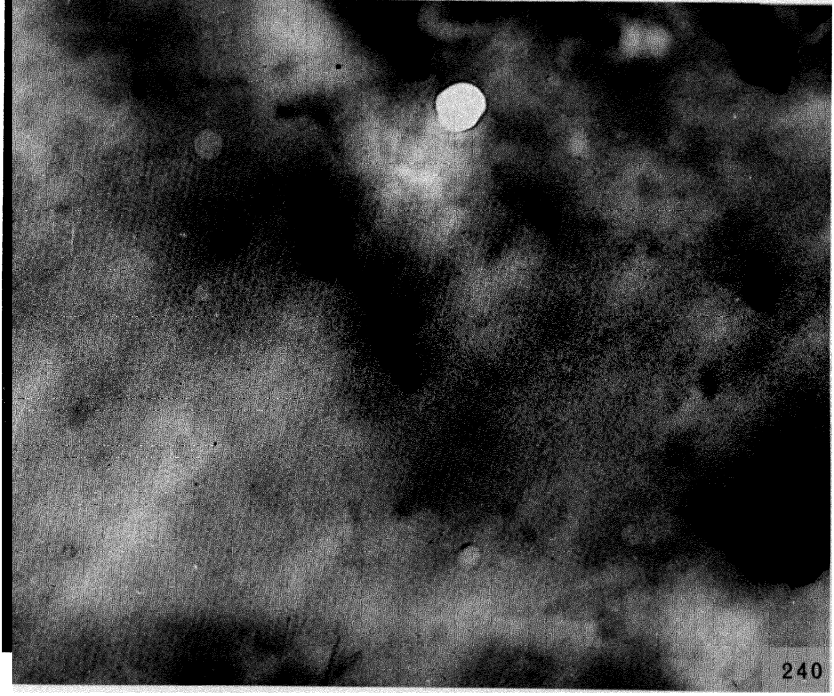
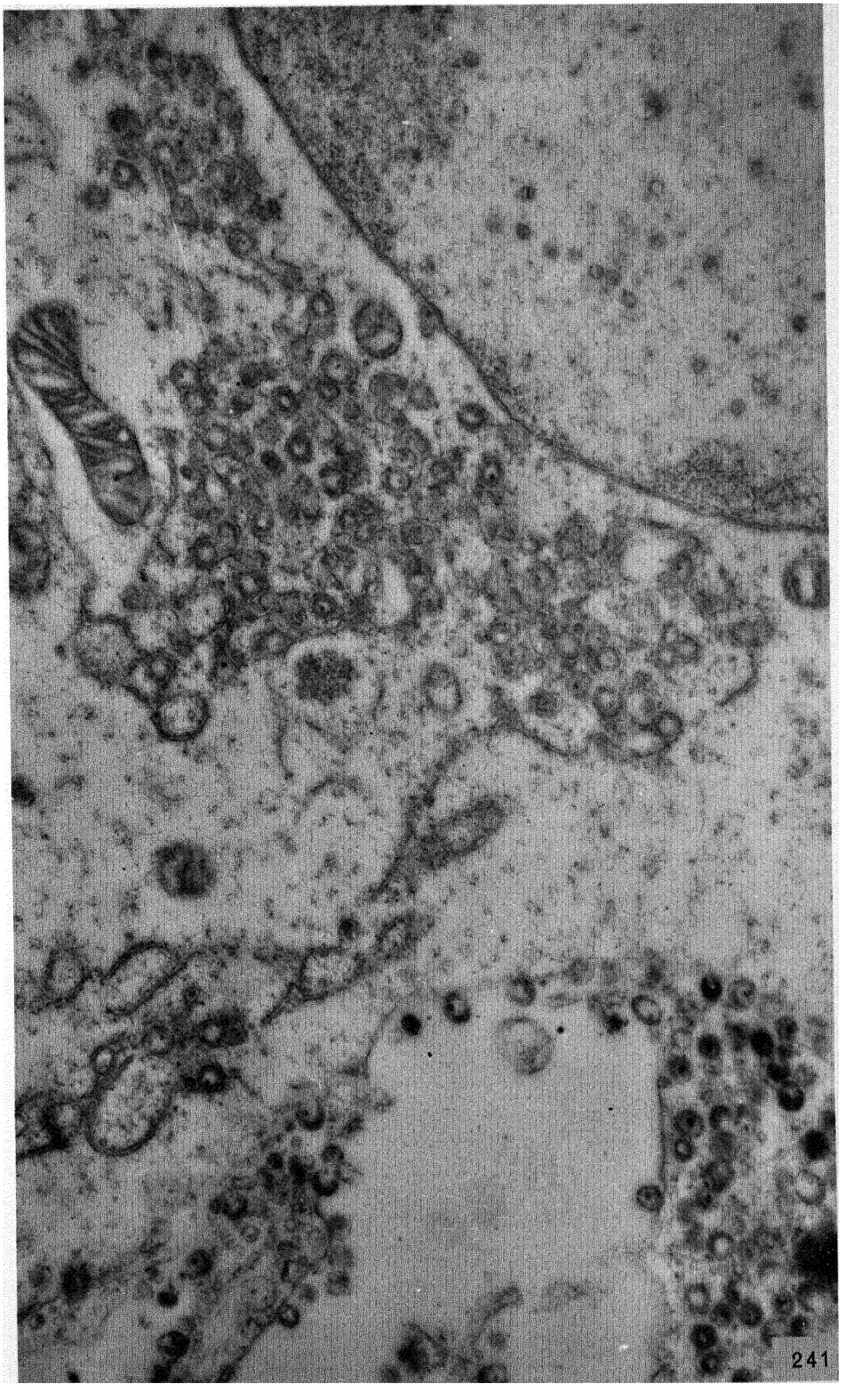


240

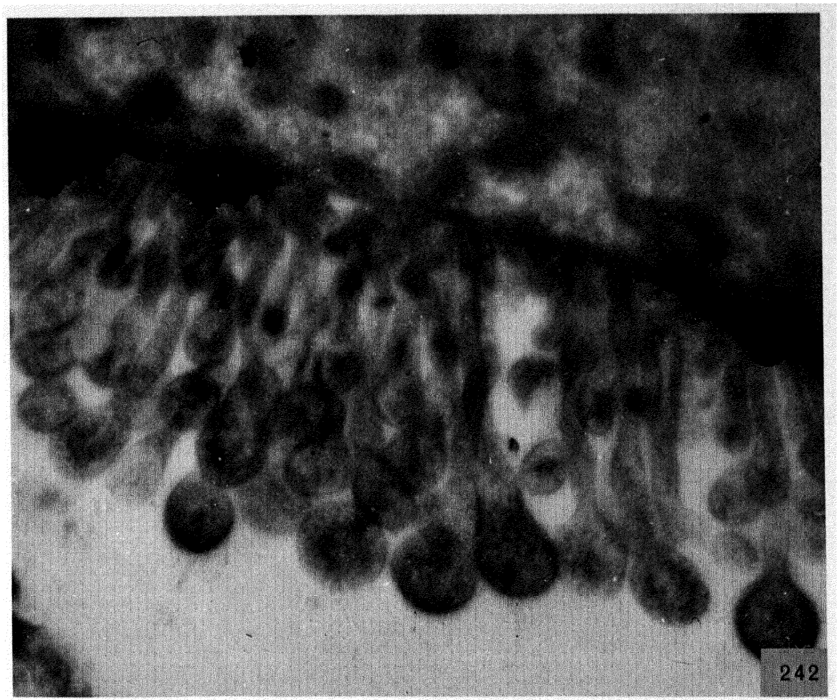
241. Herpes B virus. Virus particles originate in nucleus and develop at the expense of the chromatin. Some intranuclear particles visible upper right. More complex particles in the cytoplasm at center are typical of second stage of development and correspond to extracellular particles at bottom [57]. × 19,500.



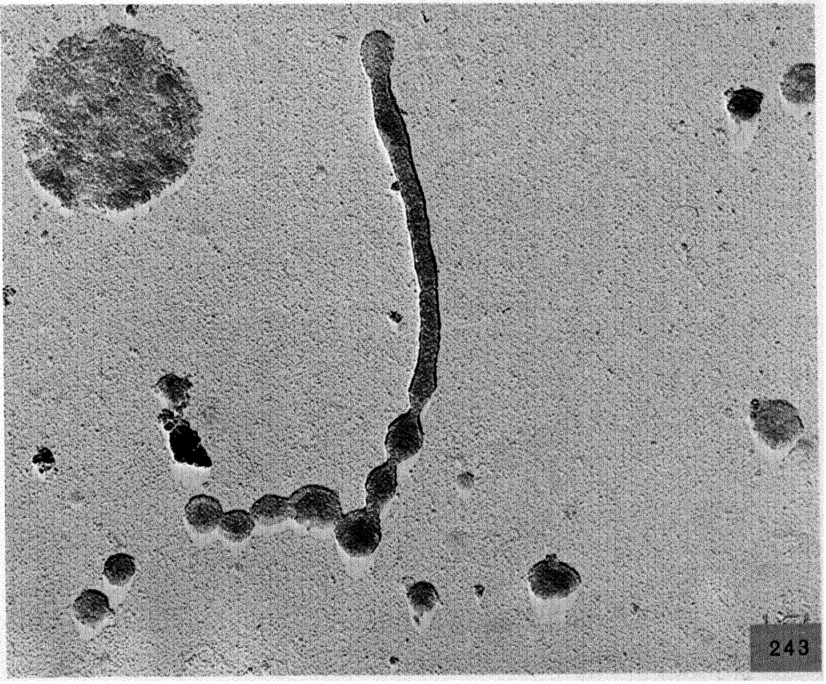
241

242. Newcastle disease virus. Long pleomorphic structures on surface of host cell typical of avirulent strains. They are either altered microvilli or "extruded" virus descending into the allantoic cavity of an egg. No sign of intracellular virus is evident. More virulent strains produce intracellular spherical particles. The closely related influenza virus produces uniform, long, thin filaments but no intracellular particles [5]. × 25,000.

243. Cell-free preparation of Newcastle disease virus. Polymorphous nature of the virus is evident. Phenomenon of particle formation from filament break-down can be seen clearly [3]. × 23,500.



242

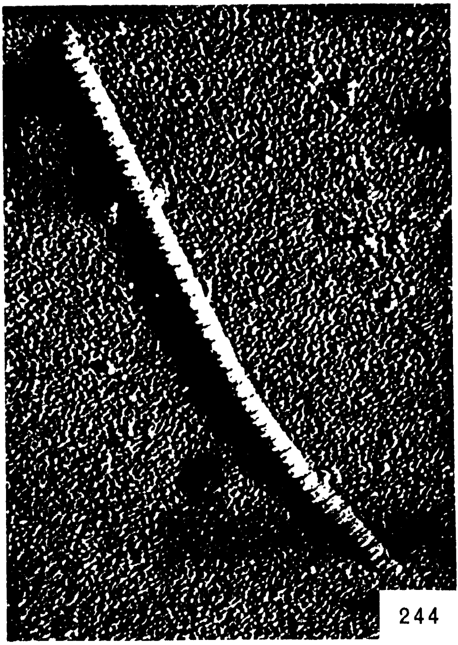


243

244. Native collagen fibril from human dura, shadowed with palladium. The broad raised bands are the A or D bands and the narrow depressed bands are the B or H bands. Two narrow bands are present in the A band and one in the B band. There is a faint longitudinal striation.

245. FLS fibril (left) and collagen fibril (right) formed by dialysis of an acid solution of collagen after the addition of mucoprotein.

246. A section of periodontal membrane which has been shadowed after removal of the embedding material. The collagen fibrils appear tubular. Note the striations in the fibril at the upper right. (Courtesy of Dr. James Kennedy, National Institutes of Health, Bethesda, Maryland)



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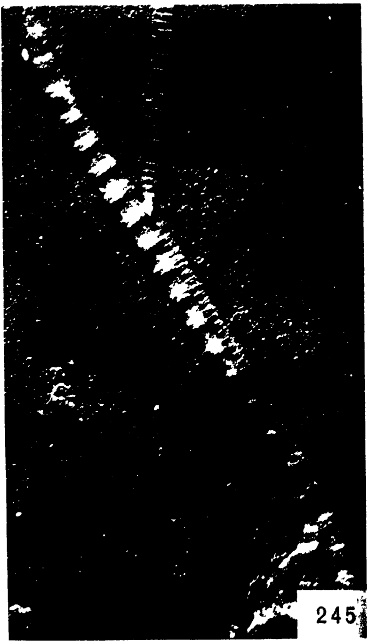

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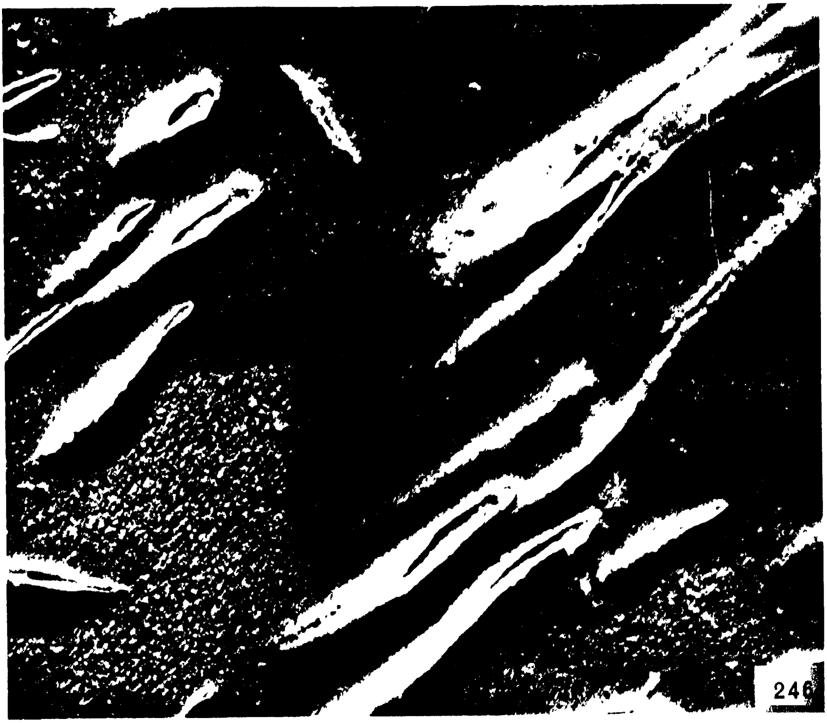
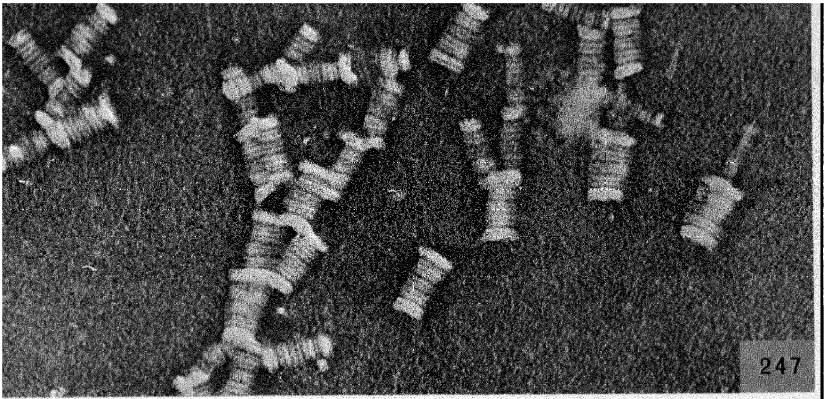


246

247. SLS fibrils. (Courtesy of Dr. Jerome Gross, Massachusetts General Hospital, Boston, Massachusetts)

248. Silver staining with internal "molecular" deposition of silver. External deposition is also present in these fibrils with the silver granules arranged along the A bands. (Picture from unpublished work of Dr. Henry Bunting)

249. Silver staining with irregular deposits of silver on the surface of the fibrils. (Picture from unpublished work of Dr. Henry Bunting)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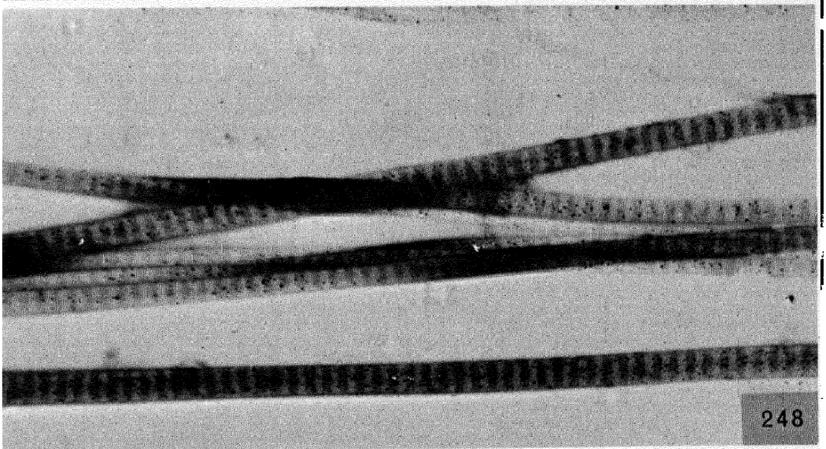
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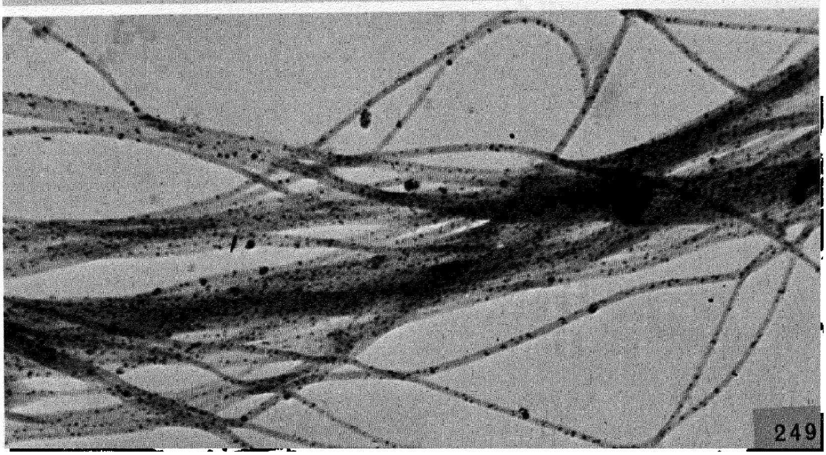
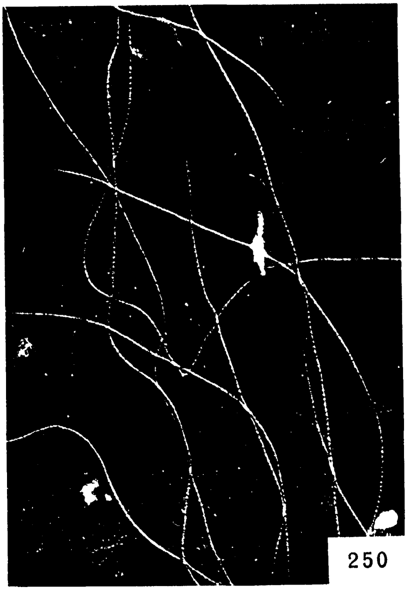

249

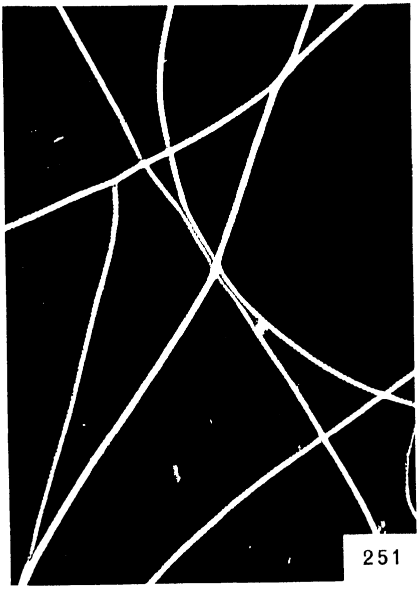
250. Collagen fibrils from the skin of a 6.8 cm fetus. This reproduction is at the same magnification as Figure 251. Note the narrow width of the fibrils and their tapered ends.

251. Collagen fibrils from the abdominal skin of a 65-year-old man. This figure is the same magnification as Figure 250.

252. Collagen fibrils after boiling. Note flattening, loss of striations and ragged margins.



250



251

252

253. Skin collagen, from a 5-year old boy, digested for 3 hours with collagenase. Both the "moth-eaten" fibrils and the beaded fibrils appear to be derived from disintegrating collagen. × 10,000. (Courtesy of Dr. Madeline Keech, Leeds University, Leeds, England [17])

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